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Binding of Chlorogenic Acid by the Isolated Polyphenol-Free 11S Protein of Sunflower (*Helianthus annuus*) Seed

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By use of a published method for the isolation of the 11S protein of sunflower seed in a homogeneous form, a procedure has been outlined for obtaining polyphenol-free 11S protein. Neither the gel filtration nor the PAGE pattern of the protein was affected by the method of polyphenol removal. The near- and far-ultraviolet circular dichroism spectrum of the protein was nearly the same as that of the protein containing polyphenols. Binding of chlorogenic acid by the polyphenol-free 11S protein of sunflower seed has been measured as a function of pH, salt concentration, and temperature. Increase in pH or salt concentration decreased the binding. Binding at 45 °C was less than at 30 °C; it was completely abolished at 55 °C. Addition of Na_2SO_3 (0.01 M), dioxane (4%), or urea (8 M) to the buffer abolished the binding. Analysis of the binding data by Scatchard equation and Hill equation showed that binding affinity was not affected by pH or salt, but the maximum number of binding sites was reduced. Decreasing the pH dissociated the 11S protein to lower molecular weight proteins. This effect was reversed by the addition of NaCl or Na_2SO_3.

INTRODUCTION

Chlorogenic acid (CGA), caffeic acid (CA), and quinic acid (QA) are the phenolic acids present in sunflower seed, the major acids being CGA and CA (70%). The main location of CGA in the seed is the aleurone or protein-containing bodies of the cell (Burns et al., 1972). Only a few papers have emphasized the practical problems of polyphenols associated with sunflower proteins. During isolation of sunflower protein, protein-containing bodies of the cell are released into the alkaline medium along with polyphenols. Polyphenolic compounds are oxidized to *o*-quinones (Pierpoint, 1969). These are highly reactive and bind in the manomeric or polymeric state covalently with thiol or amino groups of the proteins (Loomis and Battaile, 1966; Synge, 1975). Polyphenolic compounds may also react noncovalently with proteins via hydrogen bonding, ionic, and hydrophobic interactions (Loomis, 1959).

The presence of polyphenols can affect the quality of sunflower protein in several ways such as reducing the digestibility, altering the organoleptic properties, prolonging or shortening storage life and stability, and adversely altering the functional properties and behavior of sunflower protein in food systems. To minimize these problems and to devise suitable methods for sunflower protein isolation, more fundamental knowledge concerning the interactions of polyphenolic compounds with sunflower proteins is necessary.

Sunflower proteins consist of three fractions designated 11S, 7S, and 2S proteins on the basis of their sedimentation coefficients (Rahma and Narasinga Rao, 1979). The proportions of these are 70, 10, and 20%, respectively. Sabir et al. (1973) reported that CGA was bound to the low molecular weight proteins which accounted for 15% of the salt-soluble proteins in sunflower meal. Rahma (1979) reported that CGA was also associated with the 11S protein. Many methods have been reported for the isolation of the 11S protein (Joubert, 1955; Sabir et al., 1973; Schwenke and Raab, 1973; Schwenke, 1975; Rahma, 1979). However, none of these procedures yields a completely polyphenol-free protein in good yield. Various procedures have been developed for the removal of polyphenols from sunflower kernels. Extraction of sunflower kernels/meal with aqueous, organic, or aqueous–organic solvents does not give a protein completely free from polyphenols (Sosulski, 1984).

This paper deals with (i) a method to isolate polyphenol-free 11S protein in a homogeneous form and (ii) the interaction of a representative polyphenolic compound,

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chlorogenic acid, with a polyphenol-free 11S protein of sunflower seed.

MATERIALS AND METHODS

Materials. Sunflower seed of EC-68414 variety was obtained from the Karnataka Agro Industries Corp., Mysore. Sepharose 6B-100, DEAE-cellulose (coarse mesh of 0.80 mequiv/g exchange capacity), and chlorogenic acid (CGA) were from Sigma Chemical Co. All other chemicals used were of reagent grade.

Methods. *Isolation of 11S Protein.* The procedure of Rahma and Narasinga Rao (1981) was used with minor modifications. Sunflower seeds were dehulled with a centrifugal sheller (Shamanthaka Sastry, 1979). The kernels were flaked in a Kvarnmaskiner flaking machine (Model 7725) and defatted with hexane at room temperature (~30 °C). It was desolventized and milled in an apex mill to pass through BS-60 mesh. Moisture and protein contents of the meal were 6.5 and 56.3%, respectively.

The sunflower meal (10 g) was stirred with 100 mL of 10% NaCl solution for 1 h. The insoluble residue was separated by centrifugation at 3000 rpm for 20 min, and the clear supernatant was taken in a conical flask. Ammonium sulfate was added to 5% concentration, and the suspension was kept in the cold (~4 °C) for 30 min. The precipitate obtained was discarded. The ammonium sulfate concentration of the supernatant was increased to 10% and the suspension left in the cold for 30 min. The suspension was centrifuged at 4000 rpm for 20 min. The sediment was recovered and dissolved in 0.1 M Tris-HCl buffer, pH 8.3.

Gel Filtration. Sepharose 6B-100 equilibrated with 0.025 Tris–glycine buffer (pH 8.3) containing 2.5% NaCl was packed into a column, 2 cm × 85 cm. Elution of the protein was done with the same solvent. Fractions (3 mL) were collected in an automatic fraction collector, and protein was monitored by measuring the absorbance at 280 cm (Gelotte and Porath, 1967). Absorbance at 324 nm, which is a measure of CGA concentration, was also measured.

Gel Electrophoresis. This was carried out in a Shandon disc electrophoresis apparatus. Polyacrylamide gels (7.4%) were prepared in 0.025 M Tris–glycine buffer, pH 8.3. An aliquot (10 μL) containing 100 μg of protein was electrophoresed in the same buffer at 4 mA/tube for 90 min. The gels were stained with amido black and destained in 7% acetic acid for 24 h (Davis, 1964).

Absorption Spectrum. The ultraviolet absorption spectrum of the protein in 0.025 M Tris–glycine buffer, pH 8.3, was recorded in a Perkin-Elmer double-beam recording spectrophotometer 24 in the range 240–370 nm.

Florescence Spectrum. The fluorescence spectrum of the protein was measured in a Perkin-Elmer spectrofluorometer Model 203 at 25 °C. The emission spectrum of 0.01% solution in 0.025 M Tris–glycine buffer, pH 8.3, was recorded in the range 300–400 nm after excitation at 280 nm.

Circular Dichroism Spectrum. A Jasco J 20C automatic recording spectropolarimeter calibrated with *d*-10-camphorsulfonic acid was used. Protein concentration of 0.02% and cells of 0.1-cm optical path were used. The measurements were made at room temperature. Mean residue ellipticities were calculated by the standard procedure (Adler et al., 1973). A value of 115 calculated from amino acid data was used for mean residue weight (MRW).

Equilibrium Dialysis. Aliquots (2 mL) of 1% protein in the appropriate buffer were dialyzed in Visking sausage dialysis tubing for a period of 40 h at 30 ± 1 °C against 5 mL of buffer containing varying amounts of CGA. Corresponding "blanks" containing only the buffer solution were also run. All solutions contained 0.001% sodium azide to prevent microbial growth. At the end of the equilibrium period, the concentration of CGA in the solution outside the dialysis bag was estimated. From the difference in CGA concentration of the blank and the experimental, the number of CGA molecules bound by 350 000 g of protein was calculated (ν).

Protein Concentration. The concentration of 11S protein solution was determined by micro-Kjeldhal procedure. Using this protein solution of known concentration, a series of solutions in 0.1 M acetate buffer, pH 4.0, were prepared, and the absorbance of the solutions was read at 280 nm. The absorption coefficient of 1% protein solution at 280 nm, $E^{1\%}_{1cm}$, was found to be 8.2. Protein concentration was routinely estimated by absorbance measurement at 280 nm.

Concentration of Chlorogenic Acid. The concentration of CGA in water and buffer solutions was determined by measuring the absorbance at 324 nm and using a molar extinction coefficient of 1.85×10^4 (Barnes et al., 1950).

Effect of pH. To determine the effect of pH on the binding of CGA by the 11S protein, equilibrium dialysis experiments were performed at pH 4.0, 5.5 and 7.0. Acetate buffers (0.1 M) were used for pH 4.0 and 5.5, and phosphate buffer (0.1 M) was used for pH 7.0.

Effect of Ionic Strength. To assess the importance of electrostatic interactions between CGA and the 11S protein, equilibrium dialysis experiments were performed at 30 °C in 0.1 M acetate buffer of pH 4.0 containing 0.1, 0.2, 0.4, 0.6, and 1.0 M NaCl.

Reversibility of Binding. The measurements were made in 0.1 M acetate buffer at pH 4.0 at 30 °C. A mixture of protein and CGA, whose concentrations correspond to $\nu \sim 7$, was dialyzed for 40 h separately against lower concentrations of CGA and ν determined in each case.

Effect of Temperature. The effect of temperature on the binding of CGA by the 11S protein was studied by making binding measurements at 30, 45, and 55 °C in 0.1 M acetate buffer, pH 4.0.

Ultracentrifugation. A Spinco Model E analytical ultracentrifuge equipped with Schlieren optics and rotor temperature indicator and control (RTIC) unit was used. Experiments were performed with 1% protein solution in the appropriate buffer at 59 780 rpm and at 30 °C. Standard 12-mm KCl F cells were used.

Estimation of Chlorogenic Acid. This was done by the method of Pomenta and Burns (1971). Pure CGA was used as a standard.

Treatment of Data. The difference in the concentration of CGA between the control and the experimental represented the amount of ligand bound to the protein. The number of moles of CGA bound per 350 000 g of protein, ν, was plotted against the logarithm of free concentration of CGA, A. The data were analyzed by the Scatchard equation (Scatchard, 1949) to determine K, the binding constant, and N, the total number of CGA molecules bound to the protein:

$$\nu/A = NK - \nu K \quad (1)$$

The slope gives K and the intercept on the Y axis, NK. The intercept on the X axis gives N.

RESULTS AND DISCUSSION

The interaction of polyphenols with proteins is known to alter the physical, chemical, and nutritional properties of the proteins (Brown and Wright, 1963; Neucere et al., 1978; Sosulski, 1984). The study of Sabir et al. (1974) on CGA–sunflower protein interaction was done with the total proteins. It is difficult to draw meaningful conclusions on the nature of the interaction from such studies. Interaction studies are best done with isolated fractions for ease of interpretation of results, and hence purification of 11S protein free from polyphenols was necessary.

Various solvents have been used to remove polyphenols from sunflower meal (Sripad et al., 1982). However, none of them yields a protein totally free from polyphenols. It has been reported that extraction of sunflower proteins in 2% NaCl solution reduces the CGA content of the proteins to traces (Shamanthaka Sastry and Subramanian, 1984). Polyphenols undergo oxidation, and the resultant quinones interact with the proteins covalently (Loomis and Battaile, 1963). Use of reducing agents such as Na_2SO_3 has been suggested to decrease such interactions (Gheyasuddin, 1970). Sunflower meal contains metal ions

Table I. Effect of Chemical Treatment on the Chlorogenic Acid Content of Sunflower 11S Protein

treatment	CGA content, g/100 g of protein	% CGA removed
1. 11S protein dissolved in 0.1 M Tris-HCl buffer, pH 7.6, and dialyzed against the same buffer for 24 h	0.310[a]	
2. 11S protein dissolved in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.4 M NaCl and dialyzed against Tris-HCl buffer for 24 h	0.160	48
3. 11S protein dissolved in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.1 M Na_2SO_3 and dialyzed against Tris-HCl buffer for 24 h	0.048	85
4. 11S protein dissolved in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.001 M EDTA and dialyzed against Tris-HCl buffer for 24 h	0.177	43
5. 11S protein dissolved in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.4 M NaCl, 0.1 M Na_2SO_3, and 0.001 M EDTA and dialyzed against Tris-HCl buffer for 24 h	0.012	96

[a] CA and QA could not be detected.

(Robinson, 1979). It is not clear what role metal ions have in either the biological functions of the proteins or their interactions. So we tried the effect of various reagents for removal of CGA from sunflower meal. The chemicals tried were NaCl, a reducing agent (Na_2SO_3), and a metal chelating agent (EDTA). The effect of the chemicals on CGA removal is shown in Table I.

Isolated 11S protein had 0.31% CGA as compared to 0.41% of total proteins. Thus, a major portion of CGA was associated with the 11S protein. Addition of 0.4 M NaCl to the buffer solution and dialysis reduced the CGA content to 0.16%. However, the addition of 0.1 M Na_2SO_3 reduced it to 0.048%. On the other hand, the addition of 0.001 M EDTA caused a reduction to only 0.177%. Thus, none of the reagents gave a protein free from CGA. However, addition of 0.1 M Na_2SO_3, 0.4 M NaCl, and 0.001 M EDTA together to the buffer solution and dialysis gave a protein almost totally free from CGA (efficiency of removal of 96%). The mechanism by which this combination of chemicals removes CGA is not clear.

Therefore, the following procedure was used to obtain 11S protein free from CGA. The precipitate from 10% ammonium sulfate fractionation of 10% NaCl extract of sunflower meal (see Materials and Methods) was dissolved in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.4 M NaCl, 0.1 M Na_2SO_3, and 0.001 EDTA and left at room temperature for 2 h. Then it was dialyzed exhaustively against Tris buffer to remove NaCl, Na_2SO_3, and EDTA. Tests for NaCl and CGA in the buffer outside the dialysis bag showed that the reagents had been completely removed. This solution was used for experiments or dialyzed against the required buffer where necessary. The properties of polyphenol-free 11S protein (A) and polyphenol-containing 11S protein (B) were determined.

The effect of polyphenol removal on the oligomeric structure of the 11S protein was determined by gel filtration and gel electrophoresis. In gel filtration, protein A gave a single symmetrical peak (Figure 1), which eluted at the same volume (~195 mL) as protein B. In PAGE, protein A gave a single band. Also, mobility of the protein was the same as that of protein B, when run under identical conditions (Figure 2).

The ultraviolet absorption spectrum of proteins A and B indicated an absorption maximum at 278 nm and a minimum at 252 nm (Figure 3). Protein A had no absorbance around 320 nm, a region where CGA absorbs, suggesting that the protein preparation was free from CGA. On the other hand, protein B had considerable absorbance at 324 nm.

It was also of interest to determine if treatment with NaCl, Na_2SO_3, and EDTA had affected the secondary structure of 11S protein. The secondary structures of the

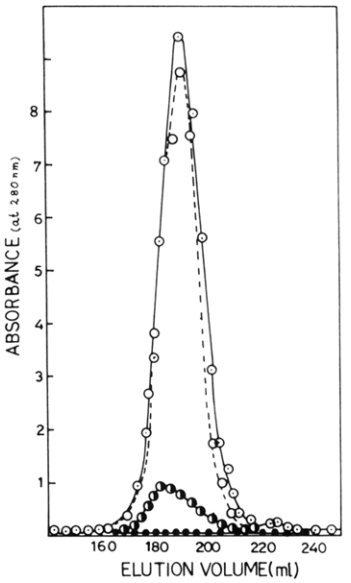

Figure 1. Gel filtration pattern of 11S sunflower protein (0.025 M Tris–glycine buffer, pH 8.3, containing 2.5% NaCl). (⊙) Absorbance at 280 nm of protein A; (○) absorbance at 280 nm of protein B; (◑) absorbance at 324 nm of protein B; (●) absorbance at 324 nm of protein A.

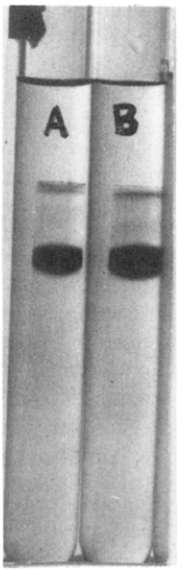

Figure 2. PAGE pattern of proteins A and B (0.025 M Tris–glycine buffer, pH 8.3).

protein were monitored by CD measurements in the far-UV region (below 260 nm). The far-UV CD spectrum of the protein showed a minimum around 208 nm with a

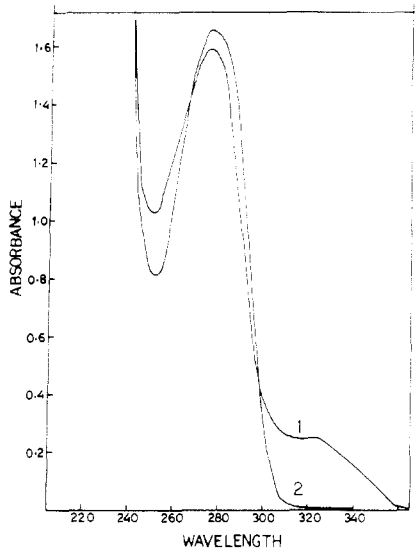

Figure 3. UV absorption spectrum of proteins A and B (0.025 Tris–glycine buffer, pH 8.3). (1) Protein B; (2) protein A.

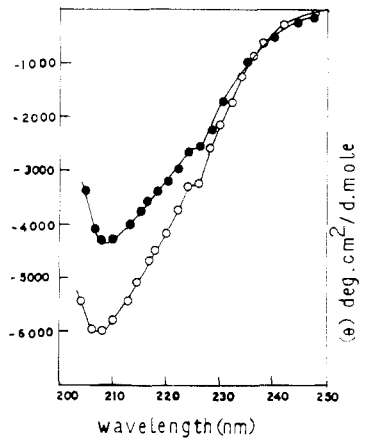

Figure 4. Far-UV CD spectrum (0.1 M phosphate buffer, pH 7.6). (●) Protein A; (○) protein B.

shoulder at 228 nm (Figure 4). The values of ellipticity at 208 and 228 nm were −4400 and −2500 (deg cm^2)/dmol, respectively, for protein A and −6000 and −3300 (deg cm^2)/dmol, respectively, for protein B.

The far-UV CD spectrum of the two proteins exhibited two minima at the same wavelength, namely, 208 and 228 nm, and the general shapes of the spectra were the same. However, there was some difference in the ellipticity values of the two proteins at the minima. The far-UV CD data have been used to calculate the secondary structure of proteins; however, such calculations are subject to serious limitations (Chang et al., 1978). Although the ellipticity value at 208 nm of the two protein differs, it is not clear if this is due to differences in the secondary structure of the proteins. CGA has absorption in the region, and this could affect the CD spectrum.

The fluorescence emission spectrum of the proteins was also determined. The two proteins had emission maximum at 325 nm (Figure 5). The intensity of protein A was slightly higher than that of protein B. CGA has strong absorption in this region, and this could have a screening effect in the case of protein B.

Sripad and Narasinga Rao (1987) have reported that removal of CGA from sunflower proteins generally enhances fluorescence emission intensity in the region 300–400 nm, although there is no correlation between the CGA content and fluorescence intensity.

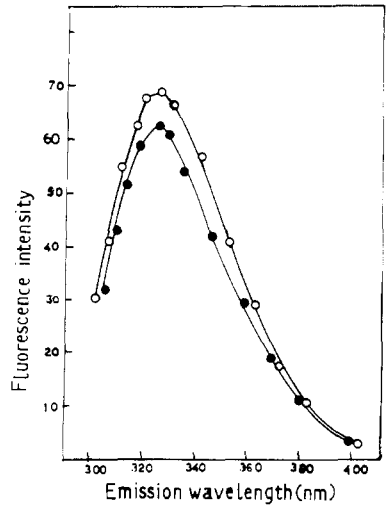

Figure 5. Fluorescence spectra of proteins A and B (0.025 M Tris–glycine buffer, pH 8.3). (○) Protein A; (●) protein B.

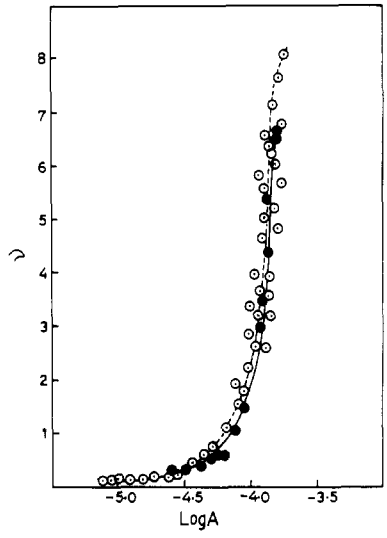

Figure 6. Binding of CGA by the 11S protein of sunflower seed (0.1 M acetate buffer, pH 4.0, 30 °C). (⊙) Direct points; (●) reversed points.

Since there was no red shift in the emission maximum, it can be concluded that treatment of the 11S protein with various chemicals to remove polyphenol had not denatured it (Cowgill, 1963; Chen et al., 1969).

Hence, the results indicate that treatment of 11S protein at pH 7.6 in Tris-HCl buffer with NaCl, Na_2SO_3, and EDTA removes more than 96% CGA and the treatment does not affect the oligomeric structure of the protein or its conformation.

Reversibility of Binding. The binding isotherm at pH 4.0 is given in Figure 6 as a plot of ν against log A. Below 1×10^{-4} M CGA there was very little binding of CGA. Above this concentration there was a sharp increase in binding, and at the highest CGA concentration used ν was ~8. Also, within the limits of experimental errors, ν values determined by the reversibility experiments fitted the same curve. This suggested that binding of CGA by 11S protein was reversible.

Binding was considerably less at pH 5.5 and 7.0 (Figure 7). The highest ν value that could be experimentally determined at pH 5.5 was ~5.0 and at pH 7.0 ~2. It was not possible to make measurements at higher concentrations of CGA since the solutions had to be diluted severalfold to bring them into the range of spectrophotometric measurements. This would introduce large errors into the calculation of ν.

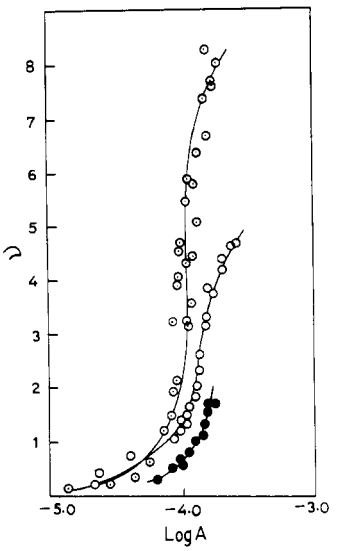

Figure 7. Binding of CGA by the 11S protein of sunflower seed at different pH values, 30 °C. (⊙) pH 4.0, 0.1 M acetate buffer; (○) pH 5.5, 0.1 M acetate buffer; (●) pH 7.0, 0.1 M phosphate buffer.

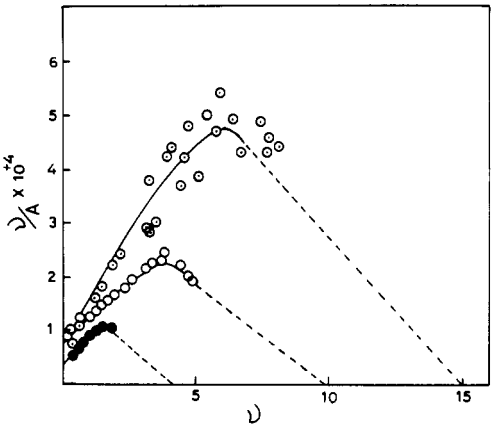

Figure 8. Scatchard plot of the binding data at different pH values. (⊙) pH 4.0, 0.1 M acetate buffer; (○) pH 5.5, 0.1 M acetate buffer; (●) pH 7.0, 0.1 M phosphate buffer; (- - -) line drawn between the maximum in the plot and N.

Analysis of Binding Data. *Scatchard Plot.* The binding data were analyzed with the Scatchard equation by plotting ν/A as a function of ν. The Scatchard plots of the binding data at pH 4.0, 5.5, and 7.0 are given in Figure 8. The plots were anomalous at all the pH values. At pH 4.0, ν/A values increased up to $\nu \sim 6$ and then showed a tendency to decrease. From the shape of the curve, it was not possible to make a reasonable extrapolation on the X axis to obtain the value of N and K.

Hill Plot. Binding that gives Scatchard plot with a positive slope is generally interpreted as exhibiting positive cooperativity of binding (Dahlquist, 1974). Such binding data can be analyzed with the Hill equation (Hill, 1910). The Hill coefficient is an index of cooperativity of binding. The Hill plot is constructed by plotting log $\nu/(N - \nu)$ as a function of log A.

Thus, a knowledge of N is necessary to construct the Hill plot. It has been shown that the Hill coefficient, n_r, is related to the maximum in the Scatchard plot, Y_m, by the relationship (Dalquist, 1974)

$$n_r = 1/(1 - Y_m) \quad (2)$$

Since the maximum in the Scatchard plot can be located reasonably well, we have used the following procedure to determine N and K.

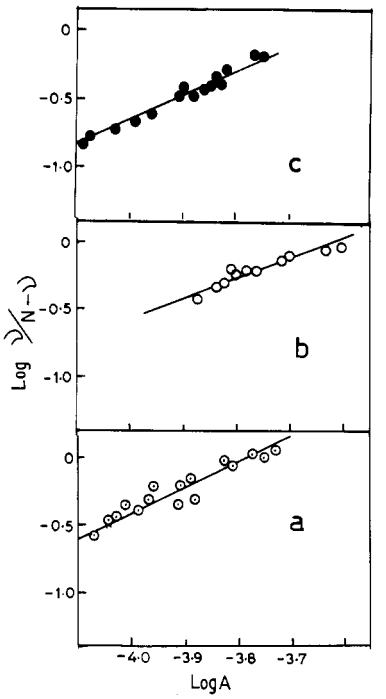

Figure 9. Hill plot of the binding data at different pH values. (a) pH 4.0, 0.1 M acetate buffer; (b) pH 5.5, 0.1 M acetate buffer; (c) pH 7.0, 0.1 M phosphate buffer.

From Figure 8, it can be seen that the data at pH 4.0 cannot yield a value of N less than 12. The data have to be strained considerably to obtain a value of N less than 12. Therefore, a set of integers above 12 was chosen, and these values were used for constructing Hill plots. The best value of N was chosen as that which gave a Hill coefficient that coincided with the value given by eq 2. To illustrate the point, the Hill plot of the binding at pH 4.0 with $N = 15$ is given in Figure 9. The slope of the plot is 1.80. The Scatchard plot of the binding data gives a maximum at $\nu = 6.5$ (Figure 3). Thus, the value of the Hill coefficient would be $1/(1 - 6.5/15) = 1.76$, a value close to 1.80. By use of this procedure the data at pH 5.5 and 7.0 were also analyzed to obtain the N values. By drawing a straight line between N and the maximum in the Scatchard plot, K was obtained from the slope. Both K and N values are given in Table II. It was observed that Hill coefficients were more sensitive to changes in N value than K value.

The results show that the N value decreased as the pH increased: the K value did not change significantly with pH. These results therefore suggest that the affinity of the protein for CGA does not change with pH. Only the number of binding sites on the protein molecule varied with pH. As the pH decreased, the value of N increased. At pH 4.0 more binding sites were available. The observation that pH had no effect on the binding constant suggested that the interaction was perhaps not ionic in nature.

Effect of NaCl. The binding measurements were made at pH 4.0 in the presence of different concentrations of NaCl. The binding isotherms are shown in Figure 10. Addition of NaCl caused a decrease in the binding of CGA by the protein.

The Scatchard plot showed that binding in the presence of NaCl also exhibited positive cooperativity. By use of the method described, the N value and Hill coefficient were determined. The Hill coefficient did not vary much: it was 1.66 at 0.1 M NaCl, 1.61 at 0.6 M NaCl, and 1.63 at 1 M NaCl. Similarly, the value of K also did not show much change; it varied from 3.1×10^3 to 3.8×10^3 (Table

Table II. Hill Coefficients and N and K Values for the Binding of CGA by 11S Protein

buffer	Hill coeff		N	K
	from slope	from Scatchard plot		
11S protein, pH 4.0 (0.1 M) (30 °C)	1.80	1.76	15	5.5×10^3
11S protein, pH 5.5 (0.1 M) (30 °C)	1.64	1.67	10	3.8×10^3
11S protein, pH 7.0 (0.1 M) (30 °C)	1.60	1.60	4	4.5×10^3
11S protein, pH 4.0 with 0.1 M NaCl (30 °C)	1.66	1.67	10	3.75×10^3
11S protein, pH 4.0 with 0.6 M NaCl (30 °C)	1.61	1.60	12	3.30×10^3
11S protein, pH 4.0 with 1.0 M NaCl (30 °C)	1.63	1.58	10	3.05×10^3
11S protein, pH 4.0 (0.1 M) (40 °C)	1.10	1.20	1.2	0.9×10^3
11S protein, pH 4.0 (0.1 M) (55 °C)				

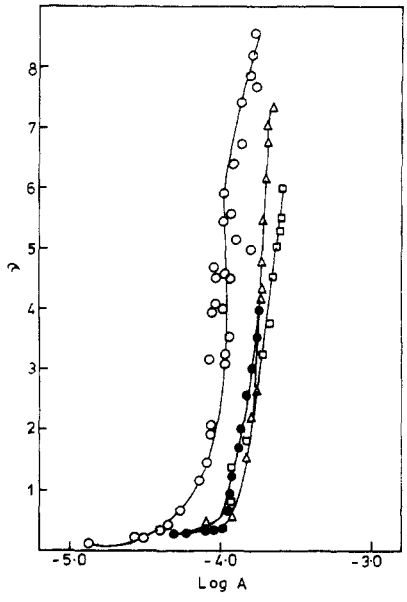

Figure 10. Effect of NaCl concentration on the binding of CGA by the 11S protein of sunflower seed at pH 4.0 (0.1 M acetate buffer) and 30 °C. (○) 0 NaCl; (△) 0.1 M NaCl; (●) 0.6 M NaCl; (□) 1.0 M NaCl.

II). The maximum number of binding sites decreased from 15 in the absence of NaCl to 10. However, the K value in the presence and absence of NaCl was nearly the same. Thus, the effect of NaCl on binding was merely to reduce the number of binding sites rather than reduce the binding affinity. The observation that binding affinity was not affected by NaCl suggested that perhaps the binding was not ionic in nature.

Effect of Temperature. At pH 4.0 the binding isotherm was determined at 30, 45, and 55 °C. Temperature had a profound effect on binding (Figure 11). At 45 °C binding was considerably reduced, and at 55 °C it was completely abolished. The Scatchard plot of the binding data at 45 °C showed positive cooperativity. The value of the Hill coefficient was 1.8; N was 1.2, and K was 0.9×10^3. Thus, all the values were considerably lower than those at 30 °C. This suggested that temperature affected not only the maximum number of binding sites but also the binding affinity. Temperature favors hydrophobic bonding and interferes with hydrogen bonding (Prakash and Nandi, 1977). The effect of temperature on binding would suggest the important role of hydrogen bonding in the binding of CGA by the 11S protein.

Higher temperatures can conceivably affect the oligomeric structure of the protein. Either dissociation or aggregation of the protein can occur. There was no precipitation of the protein or increase in the scattering of light by the solution, suggesting that aggregation had not occurred. Dissociation can lead to an *increase* in binding rather than a decrease.

Effect of Na_2SO_3. The effect of Na_2SO_3, a reducing agent, on the binding of CGA by the 11S protein at pH

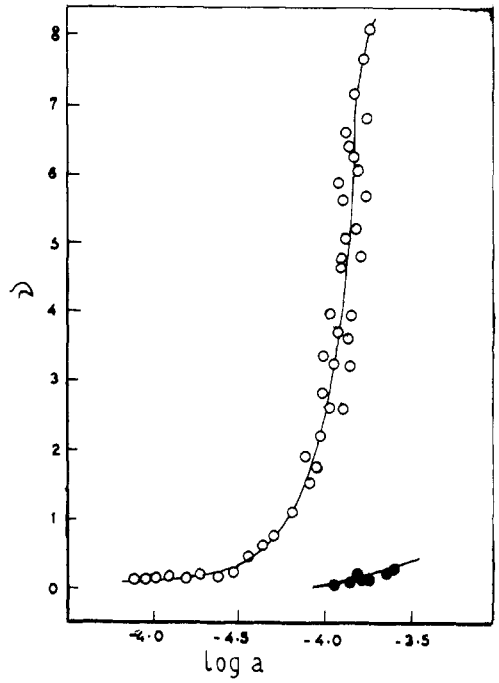

Figure 11. Effect of temperature on the binding of CGA by the 11S protein of sunflower seed at pH 4.0 (0.1 M acetate buffer). (○) 30 °C; (●) 45 °C.

4.0 was studied at two concentrations, 0.01 and 0.1 M. Binding was completely abolished at both concentrations.

Thus, both pH and salt concentration had an effect on the binding of CGA by sunflower 11S protein. Increase in pH or salt concentration (at the same pH) reduced the binding. The effect was apparently due to the decrease in the number of binding sites on the protein molecule rather than due to a decrease in binding affinity.

Sunflower 11S protein is a high molecular weight protein, its molecular weight being 350 000. It consists of 10 nonidentical subunits (Rahma and Narasinga Rao, 1981). In ultracentrifugation experiments, the protein gave a single symmetrical peak with an $s_{20,w}$ value of 11.8 at pH 7.6 (Figure 12). However, the protein was dissociated in 0.1 M acetate buffer, pH 4.0. In addition to the 11S protein it contained 2S and 7S proteins (Figure 12). The proportion of 2S, 7S, and 11S proteins was 60, 5, and 35%. Dissociation may provide more binding sites. This may explain why binding increased with decrease in pH.

It was observed that the addition of NaCl caused a decrease in the binding of CGA by the protein at pH 4.0. We have investigated the effect of NaCl on the oligomeric structure of the protein by ultracentrifugation.

The addition of 1 M NaCl to 0.1 M acetate buffer, pH 4.0, completely suppressed the dissociation of the protein at pH 4.0. The sedimentation velocity pattern consisted of a single symmetrical peak of $s_{20,w}$ = 11.8 S (Figure 13). Apparently, undissociated protein had fewer binding sites. Salts at high concentrations have been reported to prevent

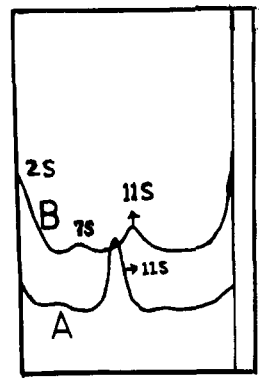

Figure 12. Sedimentation velocity pattern of the 11S protein of sunflower seed. (A) pH 7.6 (0.1 M phosphate buffer); (B) pH 4.0 (0.1 M acetate buffer). Sedimentation proceeds from left to right.

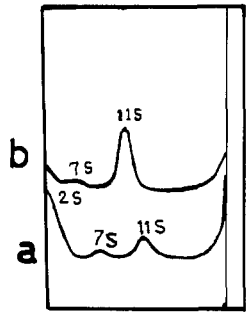

Figure 13. Sedimentation velocity pattern of the 11S protein of sunflower seed. (a) pH 4.0 (0.1 M acetate buffer); (b) pH 4.0 (0.1 M acetate buffer with 1 M NaCl). Sedimentation proceeds from left to right.

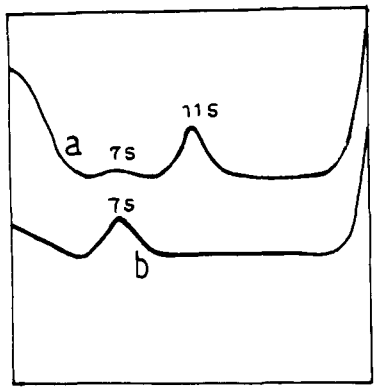

Figure 14. Sedimentation velocity pattern of the 11S protein of sunflower seed. (a) pH 4.0 (0.1 M acetate buffer); (b) pH 4.0 (0.1 M acetate buffer plus 0.01 M Na_2SO_3). Sedimentation proceeds from left to right.

the dissociation of a number of oligomeric proteins (Schwenke et al., 1975, 1978).

Even at 0.01 M concentration, Na_2SO_3 completely abolished the binding of CGA by the protein. Ultracentrifuge experiments showed that Na_2SO_3 even at 0.01 M concentration prevented the dissociation of the protein at pH 4.0. The sedimentation velocity pattern of the protein in the presence of 0.01 M Na_2SO_3 consisted of a single peak with $s_{20,w}$ = 7.2 S (Figure 14). Thus, Na_2SO_3 also prevented the dissociation of the protein. However, this does not explain why Na_2SO_3 completely abolished binding. If the effect of Na_2SO_3 was only to prevent the dissociation of the 11S protein, the binding at pH 4.0 in the presence of 0.01 M Na_2SO_3 should be the same as that at pH 7.0; this was not the case. Therefore, the effect of Na_2SO_3 appears to be more than just preventing the dissociation of the protein.

There are not many reports on the effect of Na_2SO_3 on proteins. Von Hippel and Schleich (1969) have reported that Na_2SO_3 does not cause depolymerization of F actin up to 0.6 M. $NaNO_3$ is a water structure breaker (Hatefi and Hanstein, 1969). Na_2SO_3 is more nucleophilic than $NaNO_3$ (Jencks, 1969). So it could also be a water structure breaker. This may in a complex way affect the binding of CGA by the 11S protein, which is essentially through hydrogen bonding. Also Na_2SO_3, being a reducing agent, prevents the accumulation of quinones in CGA solution (Loomis and Battaile, 1966). Quinones react covalently with proteins.

The evidence presented above indicates that the 11S protein binds CGA by hydrogen bonding. Further evidence for this conclusion was obtained by making binding measurements in the presence of dioxane, a hydrogen bond breaking reagent (Singer, 1962), and urea, which is known to break both hydrogen bonds and hydrophilic bonds (Synge, 1975). Both reagents completely abolished the binding of CGA by the protein.

ABBREVIATIONS USED

Chlorogenic acid, CGA; caffeic acid, CA; quinic acid, QA; ethylenediaminetetraacetic acid, EDTA; circular dichroism, CD.

LITERATURE CITED

Adler, A. J.; Greenfield, N. J.; Fasman, G. D. Circular Dichroism and optical rotatory dispersion of proteins and polypeptides. *Methods Enzymol.* **1973**, *27D*, 675.

Barnes, H. M.; Feldmann, J. R.; White, N. V. Isochlorogenic acid; Isolation from Coffee and structure studies. *J. Am. Chem. Soc.* **1950**, *72*, 4178.

Brown, P. J.; Wright, W. B. An investigation of the Interaction between Milk proteins and Tea polyphenols. *J. Chromatogr.* **1963**, *11*, 504.

Burns, E. E.; Talley, L. J.; Brummett, B. Sunflower utilisation in human foods. *Cereal Sci. Today* **1972**, *17*, 287.

Chang, C. T.; Wu, C. C.; Yang, J. T. Circular Dichroic Analysis of protein conformation inclusion of the B-turns. *Anal. Biochem.* **1978**, *91*, 13.

Chen, R. F.; Edelhoch, H.; Steiner, R. F. In *Physical principles and techniques of protein chemistry*; Leach, S. J., Ed.; Academic Press: New York, 1969; Part A, p 171.

Cowgill, R. W. Fluorescence and the structure of proteins II: Fluorescence of peptides containing tryptophan of tyrosine. *Biochim. Biophys. Acta* **1963**, *75*, 272.

Dalquist, F. W. The quantitative interpretation of maximum in Scatchard plots. *FEBS Lett.* **1974**, *49*, 267.

Davis, B. J. Disc Electrophoresis 2: Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **1964**, *121*, 404.

Gelotte, B.; Porath, J. In *Chromatography*; Hoffman, E., Ed.; Reinhold: New York, 1967.

Gheyasuddin, S. Studies of the proteins and the polyphenols of Sunflower seed. Ph.D. Dissertation, Texas A&M University, College Station, TX, 1970.

Gheyasuddin, S.; Cater, C. M.; Mattil, K. F. Preparation of a colourless sunflower protein isolate. *Food Technol.* **1970**, *24*, 242.

Hatefi, Y.; Houstein, W. G. Solubalisation of particulate proteins and non-electrolytes by chaotropic agents. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *62*, 1129.

Hill, A. V. *J. Physiol.* **1910**, *90*, 4.

Jencks, W. P. In *Catalysis in Chemistry and Enzymology*; McGraw Hill: New York, 1969; p 91.

Joubert, F. J. Sunflower seed proteins. *Biochim. Biophys. Acta* **1955**, *16*, 520.

Loomis, W. D. Amide metabolism in higher plants, III: Distribution of glutamyl transferase and glutamine systhetase activity. *Plant Physiol.* **1959**, *34*, 541.

Loomis, W. D.; Battaile, J. Biosynthesis of Terpenes: III: Mevalonic kinase from higher plants. *Biochim. Biophys. Acta* **1963**, *67*, 54.

Loomis, W. D.; Battaile, J. Plant phenolic compounds and the isolation of plant enzymes. *J. Phytochem.* **1966**, *5*, 423.

Neucere, N. J.; Jacks, T. J.; Sumrell, G. Interactions of globular protein with simple polyphenols. *J. Agric. Food Chem.* **1978**, *26*, 214.

Pierpoint, W. S. O-Quinones formed in plant extracts—their reactions with amino acid and peptides. *Biochem. J.* **1969**, *112*, 609.

Pomenta, J. V.; Burns, E. E. Factors affecting chlorogenic, quinic and caffeic acid levels in sunflower kernels. *J. Food Sci.* **1971**, *36*, 490.

Prakash, V.; Nandi, P. K. Association–Dissociation and Denaturation behaviour of an oligomeric seed protein, O-globulin of *Sesamum indicum l* in acid and alkaline solution. *Int. J. Pept. Protein Res.* **1977**, *9*, 319.

Rahma, E. H. Characterisation and Functional properties of the proteins of sunflower seed. Ph.D. Dissertation, University of Mysore, Mysore, India, 1979.

Rahma, E. H.; Narasinga Rao, M. S. Characterisation of sunflower proteins. *J. Food Sci.* **1979**, *44*, 579.

Rahma, E. H.; Narasinga Rao, M. S. Isolation and characterisation of the major protein fraction of Sunflower seeds. *J. Agric. Food Chem.* **1981**, *29*, 518.

Robinson, F. G. *Agron. J.* **1979**, *67*, 579.

Sabir, M. A.; Sosulski, F. W.; Machenzie, S. L. Gel Chromatography of sunflower proteins. *J. Agric. Food Chem.* **1973**, *21*, 988.

Sabir, M. A.; Sosulski, F. W.; Kernan, J. A. Phenolic constituents of sunflower flour. *J. Agric. Food Chem.* **1974**, *22*, 572.

Scatchard, G. *Ann. N.Y. Acad. Sci.* **1949**, *51*, 660.

Schwenke, K. D. Plant seed globulins as dissociation and association systems. *Nahrung* **1975**, *19*, 69.

Schwenke, K. D.; Raab, B. On seed proteins: Part I: The distribution of the protein fractions obtained from sunflower seeds. *Nahrung* **1973**, *17*, 373.

Schwenke, K. D.; Schultz, M.; Linow, K. J. Dissociation behaviour of 11S globulin from sunflower seeds. *Nahrung* **1975**, *19*, 425.

Schwenke, K. D.; Sologub, L. P.; Braudo, E. E.; Tolstoguzov, W. B. Influence of neutral salts on the solubility of 11S globulin from Sunflower seeds. *Nahrung* **1978**, *22*, 491.

Shamanthaka Sastry, M. C. Studies on Sunflower proteins. M.Sc. Dissertation, University of Mysore, Mysore, India, 1979.

Shamanthaka Sastry, M. C.; Subramanian, N. Preliminary studies on processing of Sunflower seed to obtain sunflower edible protein concentrates. *J. Am. Oil Chem. Soc.* **1984**, *61*, 1039.

Singer, S. J. The properties of proteins in non-aqueous solvents. *Adv. Protein Chem.* **1962**, *17*, 1.

Sosulski, F. W. In *Food Proteins III*; Hudson, B. J. F., Ed.; Elsevier: London, 1984; p 113.

Sripad, G.; Narasinga Rao, N. S. Effects of acid pH on the 11S protein of Sunflower seed. *J. Agric. Food Chem.* **1987**, *35*, 668.

Sripad, G.; Prakash, V.; Narasinga Rao, M. S. Extractability of polyphenols of sunflower seed in various solvents. *J. Biosci.* **1982**, *4*, 145.

Synge, R. L. N. Interactions of polyphenols with proteins in plant and plant products. *Qual. Plant.—Plant Foods Hum. Nutr.* **1975**, *24* (3/4), 337.

Von Hippel, P. H.; Schleich, T. In *Structure and stability of biological Macromolecules*; Timasheff, S. N., Fasman, G. D., Eds.; Dekker: New York, 1969; p 559.

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