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Interaction of 3′-O-caffeoyl D-quinic acid with multisubunit protein helianthinin†

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Abstract. Chlorogenic acid, 3'-O-caffeoyl D-quinic acid, is an inherent ligand present in *Helianthus annuus* L. The effect of pH on chlorogenic acid binding to helianthinin suggests that maximum binding occurs at pH 6·0. The protein-polyphenol complex precipitates as a function of time. The association constant of the binding of chlorogenic acid to helianthinin, determined by equilibrium dialysis, at 31°C has a value of $3{\cdot}5 \pm 0{\cdot}1 \times 10^4 M^{-1}$ resulting in a ΔG value of – 6·32 ±0·12 kcal /mol. The association constant K_a is $1{\cdot}0 \pm 0{\cdot}1 \times 10^4 M^{-1}$ as determined by ultraviolet difference spectral titration at 25°C with ΔG^o of -5·46 ± 0·06 kcal/mol. From fluorescence spectral titration at 28°C, the K_a value is 1·38 ± 0·1 × 1 0 $4M^{-1}$ resulting in a ΔG of – 5·70 ± 0·05 kcal/mol. The total number of binding sites on the protein are 420 ± 50 as calculated from equilibrium dialysis. Microcalorimetric data of the ligand-protein interaction at 23°C suggests mainly two classes of binding. The thermal denaturation temperature, T_m of the protein decreases from 76°C to 72°C at 1×10^{-3}M chlorogenic acid concentration upon complexation. This suggests that the complexation destabilizes the protein. The effect of temperature on K_a of chlorogenic acid shows a nonlinear increase from 10·2°C to 45°C. Chemical modification of both lysyl and tryptophanyl residues of the protein decreases the strength of binding of chlorogenic acid. Lysine, tryptophan and tyrosine of protein are shown to be present at the binding site. Based on the above data, it is suggested that charge-transfer complexation and entropically driven hydrophobic interaction are the predominant forces that are responsible for binding of chlorogenic acid to the multisubunit protein, helianthinin.

Keywords. Chlorogenic acid; 3'-O-caffeoyl D-quinic acid; helianthinin; interaction.

1. Introduction

Proteins are known to interact with small ligands among which polyphenols play a significant role. The exact mechanism of interaction, though difficult to pinpoint, has been shown in single polypeptide chains as due to the covalent linkage of lysyl residues with the phenolic ring of the ligand (Pierpoint 1969a,b). The non-covalent interaction of polyphenols with monomeric and multimeric proteins is less understood (Loomis and Battaile 1966). And no detailed studies are available to enunciate thermodynamics and mechanism of the interaction of 3'-O-caffeoyl D-quinic acid with the multisubunit proteins.

Several phenolic components are present in sunflower seeds (Sabir *et al* 1974). The major polyphenols are chlorogenic acid (CGA) and caffeic acid (3,4-dihydroxy cinnamic acid). Figure 1 shows the structure of CGA which has two rings, an

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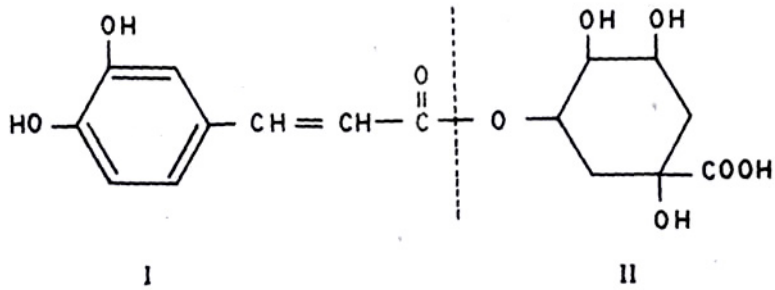

Figure 1. Structure of chlorogenic acid. It is composed of (I) 3,4-dihydroxy cinnamic acid and (II) l',3',4',5' -tetra hydroxy cyclohexane carboxylic acid.

aromatic dihydroxy phenolic ring and a non-aromatic quinic acid ring, separated by a hydrophobic acrylyl spacer. Polyphenols are known to decrease the nutritive value of important forage crops (Haslam and Lilley 1988). Polyphenols reduce the digestibility of food items by binding to the digestive enzymes (Feeny 1969; Mole and Waterman 1986). In plants, polyphenols play a defensive role by binding mostly at the active sites of enzymes of physiological importance (Goldstein and Swain 1965; Kumar and Singh 1984; Haslam and Lilley 1988; Ludlum *et al* 1991).

Helianthus annuus seed contains two major protein fractions namely, helianthinin and conhelianthinin, with sedimentation coefficient values of 11S and 2S, respectively. Out of these, helianthinin constitutes nearly 60% of the total protein (Prakash and Rao 1986). Helianthinin has been isolated and characterized by several workers (Prakash and Rao 1986). However most of the protein preparations contain polyphenols (inherently present in the seed) which interact during the process of isolation depending upon the method of isolation (Rahma and Rao 1981; Prasad 1990). Helianthinin is a multisubunit protein having six acidic and six basic subunits with acidic and basic subunits held together by disulphide linkages (Prakash and Rao 1986). Helianthinin retains its native structure in the pH range 4·2–8·0, outside which the quaternary structure is disrupted. Empirically, interaction of CGA with helianthinin has been attempted but no detailed data is available on the mechanism and thermodynamics of interaction of this protein with CGA. Hence, in the present investigation, interaction between CGA and helianthinin, a multisubunit protein has been studied from a thermodynamic and mechanistic point of view to understand the mechanism of interaction of polyphenols with multisubunit proteins.

2. Materials and methods

2.1 *Materials*

H. annuus seeds of Morden variety were purchased from Karnataka State Seeds Corporation, Mysore. CGA, Sepharose 6B-100, succinic anhydride, 2,4,6-trinitrobenzene sulphonic acid (TNBS), N-bromosuccinimide (NBS), N-acetyl L-tryptophan ethyl ester, N-acetyl L-tyrosine ethyl ester and l-anilino-8-naphthaIene sulphonic acid (ANS) were purchased from Sigma Chemical Company, St. Louis, Mo, USA;

sodium sulphite was from Ranbaxy Laboratories Ltd., Punjab; sodium phosphates (monobasic and dibasic) were from E Merck (India) Ltd., Bombay; sodium chloride was from Qualigens Fine Chemicals, Bombay; sodium azide was from Loba-Chemie, Bombay. All reagents used were of analytical grade.

2.2 *Isolation of helianthinin*

H. annuus seed total proteins were extracted from the defatted flour in 0·02M phosphate buffer (PB) containing 1 M sodium chloride and 0·02% sodium sulphite at pH 5·8. The supernatant was precipitated with 20% (w/v) ammonium sulphate at room temperature. The precipitate was dissolved in the above buffer containing 0·02% sodium azide, dialyzed against the same buffer and centrifuged. The supernatant obtained was chromatographed on a 90 × 2·5 cm Sepharose 6B-100 column at a flow rate of 20 ml/h. The peak eluting immediately after void volume was collected, dialyzed free of salts and lyophilized. The protein was periodically checked for amino acid composition and correlation coefficient obtained was >0·99 between preparations of helianthinin.

2.3 *Protein concentration*

Concentration of helianthinin was routinely determined by taking the absorbance at 280 nm and using an $E^{1\%}_{1cm}$ value of 8·8 which is estimated as follows. Approximately 10mg/ml of clear helianthinin solution was digested with concentrated sulphuric acid and its nitrogen content estimated according to AOAC (1984) procedure. Nitrogen content is multiplied by an empirical factor of 6·25 to obtain the protein content. The absorbance of the same solution after dilution was accurately measured in a Shimadzu UV-150-01 spectrophotometer. Then the absorbance is correlated with the protein content to give $E^{1\%}_{1cm}$.

2.4 *CGA concentration*

Concentration of CGA was routinely calculated by converting the absorbances of Solutions using a molar extinction coefficient of $1{\cdot}85 \times 10^4\ M^{-1}\ cm^{-1}$ at 325 nm and pH 6·0 (Barnes *et al* 1950). Molar extinction coefficient of CGA at different pH values was calculated by transferring the known amount of neutral CGA solution to different final pH values and measuring its absorption.

2.5 *Analytical ultracentrifugation*

2.5a *Sedimentation velocity:* Sedimentation velocity experiments of helianthinin were carried out at 2,59,700 g in a Spinco Model E Analytical Ultracentrifuge (Beckman, USA) equipped with phase plate schlieren optics and a rotor temperature indicator and control unit. A protein concentration of 7·8 mg/ml in 0·02M PB containing 1 M NaCl at pH 6·0 was used. In another experiment, helianthinin equilibrated with 1×10^{-4} M CGA in 0·25 M PB, pH 6·0 was centrifuged. Runs were carried out using 12 mm Kel F centerpiece using An D rotor. Photographs were taken at regular intervals of time and were read using a Gaertner

Microcomparator M-2000 interfaced with a Gaertner dual axis X-Y digital display unit. The sedimentation coefficient was calculated according to the standard procedure (Schachman 1959).

2.5b *Sedimentation equillibrium:* Helianthinin at a concentration of $9{\cdot}13 \times 10^{-7}$ M in 0·15 M PB (pH 6·0) alone and in the presence of $7{\cdot}46 \times 10^{-6}$ M CGA concentration were subjected to sedimentation equilibrium at 12,280 *g* and monitored at 280 nm and 325 nm using absorption optics. The data was analysed by curve fitting procedures for the determination of molecular weight.

2.6 *Effect of pH on CGA-helianthinin interaction*

To helianthinin of $2{\cdot}35 \times 10^{-6}$ M in different pH values, CGA was added to a final concentration of either $9{\cdot}6 \pm 0{\cdot}1 \times 10^{-5}$M or $9{\cdot}8 \pm 0\ 1 \times 10^{-4}$M, shaken and incubated for 1 h at room temperature. Different buffer systems used were (i) pH 1·6 and 2·1 (KCl-HCl buffer), (ii) pH 2·9 and 3·3 (citrate buffer), (iii) pH 4·0 and 5·0 (acetate buffer), (iv) pH 6·2, 7·2 and 8·0 (phosphate buffer), (v) pH 9·0 (Tris buffer) and (vi) pH 10·0 (carbonate buffer). The mixture was centrifuged at 10,000g for 20 min and the supernatant was analysed for CGA content. Appropriate corrections were given for the absorbance of CGA at different pH values.

2.7 *Spectral titration studies with CGA at pH 6·0*

Precipitation is negligible in 0·15 M phosphate buffer, pH 6·0 when compared to 0·02 M phosphate buffer, pH 6·0 (which was used for the pH dependent precipitation study described above). Hence for all the binding experiments, 0·15 M phosphate buffer is used to maintain the solution state.

2.7a *Difference spectral titration:* Helianthinin solution of $3{\cdot}18 \times 10^{-6}$M in 0·15 M PB (pH 6·0) was taken in tandem cuvettes and CGA was added to a final concentration range of $3{\cdot}16 \times 10^{-6}$M to $1{\cdot}88 \times 10^{-4}$ M essentially according to the setup given by Kronman and Robbins (1970). The difference spectra were recorded in the range of 200–400 nm within 1 min after mixing at 25°C. In another set of experiment at 25·0±0·5°C, CGA of $5{\cdot}41 \times 10^{-6}$M was titrated with helianthinin and change in molar extinction coefficient, ΔE was calculated (Oberfelder and Lee 1985).

From the ΔE values, mol of ligand bound per mol of protein, γ, and free ligand concentration, C_L were calculated for the difference in absorbance data obtained at 325 and 240 nm (Oberfelder and Lee 1985). Total number of binding sites, *n,* were evaluated from Klotz plot (Klotz *et al* 1946). Hill coefficient, n_H was calculated from Hill plot. The association constant, K_a was calculated to be the reciprocal of the ligand concentration at which 50% of maximal binding occurred (Dahlquist 1978). These initial values of *n,* n_H and K_a were used to obtain better refined parameters by Monte Carlo analysis to fit the experimental curve. The binding equation used (Cantor and Schimmel 1980) is:

$$\gamma = \frac{n(K_a)^{n_H}\ (C_L)^{n_H}}{1 + (K_a)^{n_H}\ (C_L)^{n_H}}\ . \qquad (1)$$

2.7b *Fluorescence spectral titration:* Fluorescence emission spectra of $4{\cdot}7 \times 10^{-7}$ M helianthinin in 0·15 M PB (pH 6·0), upon addition of CGA in the concentration range $3{\cdot}38 \times 10^{-7}$ M to $1{\cdot}10 \times 10^{-4}$ M, were recorded in the wavelength range 300–400 nm using a Shimadzu RF-5000 Recording Spectrofluorophotometer. The protein was excited at 285 nm. The different temperatures used were 10·2°C, 20°C, 28·0°C, 35·0°C, 40·2°C and 45·0°C with an accuracy of ±0·2°C.

Inner filter effects due to sample absorbance at excitation wavelength were corrected by the following equation (Birdsall *et al* 1983):

$$F_{obs} = F_{corr} \times [(e^{-aL,d} - e^{-aL},) / aL, (1 - d)], \tag{2}$$

where F_{obs} is the observed fluorescence intensity of the protein, F_{corr} is the fluorescence intensity of the protein after inner filter effect correction, a is the molar extinction coefficient of CGA, L_t is the total concentration of CGA (M) and d is the fraction of the total path length (taken as 0·5). Inner filter effect correction was also given for absorbance at emission wavelength. This correction was given for the entire wavelength region at an interval of 5 nm in the range of 300–400 nm and 2 nm interval at the peak. Association constant was evaluated using the equation (Lehrer and Fasman 1966):

$$\beta/1 - \beta = K_\alpha C_L, \tag{3}$$

where β is fractional binding and C_L is free ligand concentration (M) with C_L being evaluated using the equation

$$C_L = C^o_L - ß\ C^o_p \tag{4}$$

where C^o_L is the total ligand concentration and C^o_p is the total protein concentration. Total number of binding sites were evaluated from a plot of 1/ 1– β versus C^o_L/ß (Ward 1985).

In another experiment polarization measurements of the protein in the presence and absence of CGA were performed using a Hitachi F-4010 spectrofluorometer using 1 cm pathlength quartz cuvettes. Polarizability (P) values were calculated from the following equation

$$P = (I_{vv} - G\ I_{vh}) / (I_{vv} + G\ I_{vh}), \tag{5}$$

where G is equal to I_{hv} /I_{hh}. I_{vv} and I_{vh} are the fluorescence intensities with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. I_{hh} is the fluorescence intensity with both the excitation and emission polarizers kept in horizontal orientation.

2.8 *Equilibrium dialysis*

Binding of CGA to helianthinin at pH 6·0 was performed by equilibrium dialysis at 31·0±0·5°C. Helianthinin solution (0·5 ml of $1{\cdot}04 \times 10^{-5}$M) was taken in dialysis bag and allowed to equilibrate in 5·0 ml of CGA solutions of desired concentration along with proper blanks for 12h. After equilibration the concentration of CGA

in the outside solution was determined by measuring its absorbance at 325 nm. The difference in CGA concentrations between control and sample is taken as bound ligand concentration (Steinhardt and Reynolds 1969). Subsequently, binding isotherm and Scatchard plot (Scatchard 1949) were obtained. Initial estimate of the association constant was taken as the ligand concentration at which 50% of maximum binding occurred. Hill coefficient was calculated from Hill plot of the data. Total number of binding sites were calculated from Klotz plot (Klotz *et al* 1946). Then using these estimates from the graphical extrapolations the curve is simulated by Monte Carlo analysis through which better binding parameters were obtained.

2.9 *Circular dichroism*

Circular dichroic spectra of $1{\cdot}65 \times 10^{-6}$ M helianthinin in 0·15 M PB (pH 6·0) in the presence and absence of CGA were recorded using a Jasco J 20-C Spectropolarimeter at 25 ± 1°C. The molar ellipticities were calculated using a mean residue weight of 113 for helianthinin obtained from its amino acid composition. The secondary structure content of helianthinin was calculated by the software, CDESTIMA (courtesy: Prof. G D Fasman, Brandeis University, Waltham, Massachusetts, USA).

2.10 *Thermal denaturation*

The thermal denaturation profile of helianthinin in the presence ($8{\cdot}88 \times 1\ 0^{-5}$ M and 1×10^{-3}M) and absence of CGA was monitored using Gilford II Response Spectrophotometer. The absorbance was monitored at 287 nm in the temperature range 25–95°C in steps of 1°C. The denaturation curve was analysed by taking first derivative plots and picking the denaturation temperature, T_m using the software provided with the instrument.

2.11 *Microcalorimetry*

The thermodynamic parameters of the interaction of CGA with helianthinin were obtained from titration using Omega Microcalorimeter. The Microcalorimeter cell was equilibrated for more than 65 h at 23°C. Internal calibration was performed for cell constants and other parameters of the Microcalorimeter. A protein concentration of $7{\cdot}27 \times 10^{-6}$ M was taken in the reaction vessel. CGA at a concentration of $2{\cdot}41 \times 10^{-4}$M was automatically delivered through the computer controlled syringe at a constant injection schedule of 15 µl/min at an interval of 4 min between injections for a total of 15 injections. Using the Origin software provided with the instrument different classes of binding sites were identified.

2.12 *Interaction of CGA with chemically modified helianthinin*

Lysyl residues of the protein helianthinin were modified by succinylation at pH 8·0. Succinic anhydride was added in small increments while keeping the pH constant (Klotz 1967). The protein was dialyzed against Water and lyophilized. The extent of succinylation of protein was assessed by using 2,4,6,-trinitro benzene

sulphonic acid (Hall *et al* 1973). Binding of CGA with succinylated protein was followed at 28°C by fluorescence spectroscopy as described earlier.

Exposed tryptophan groups of the protein were modified at pH 4·0 in acetate buffer by adding 9×10^{-3} M N-bromosuccinimide in small aliquots (Spande and Witkop 1967), dialyzed against water and lyophilized. Binding of CGA with tryptophan modified protein was followed at 28°C by fluorescence spectroscopy as described earlier.

2.13 *NBS protection experiments*

CGA was added to the native and succinylated protein to form the complex at pH 6·0 and then the solution was titrated with NBS. The number of modified tryptophan groups were calculated from which the number of tryptophan residues protected towards NBS modification as a result of binding of CGA was obtained.

2.14 *Interaction of CGA with model compounds*

N-acetyl L-tryptophan ethyl ester was titrated with CGA at 28°C and the fluorescence intensity of tryptophan at 355 nm was measured. Similarly, N-acetyl L-tyrosine ethyl ester was titrated with CGA at 28°C and the fluorescence intensity of tyrosine at 308 nm was followed. Concentration of tryptophan and tyrosine taken was $1{\cdot}82 \times l0^{-5}$ M and $7{\cdot}5 \times 10^{-5}$ M, respectively. The fluorescence intensity was corrected for the inner filter effects according to the procedure described earlier. The corrected quenching data was analysed for the association constant and stoichiometry.

3. Results and discussion

It is necessary to characterize both the protein and CGA for homogeneity/purity and its spectral properties before undertaking an interaction study. Helianthinin isolated by the method described is homogeneous by both sedimentation velocity and PAGE pattern. It has an absorption maximum at 279 nm and fluorescence emission maximum at 332 nm. CGA has absorption maxima at 325 nm at pH 6·0.

3.1 *pH dependency of the interaction*

Initially precipitation of helianthinin-CGA complex has been studied as a function of pH in order to find the pH of maximum complexation. In figure 2 is shown the effect of pH on per cent CGA remaining in supernatant after helianthinin-CGA complexation. The degree of precipitation of helianthinin-CGA complex was more at pH 6·0–7·0 than at pH 3·0–4·0. The amount of precipitate increases with increase in both CGA and protein concentration at pH 6·0 in 0·02M phosphate buffer. However, the precipitation is negligible in 0·15M phosphate buffer at pH 6·0. Hence, further studies of interaction of helianthinin with CGA have been carried out at pH 6·0.

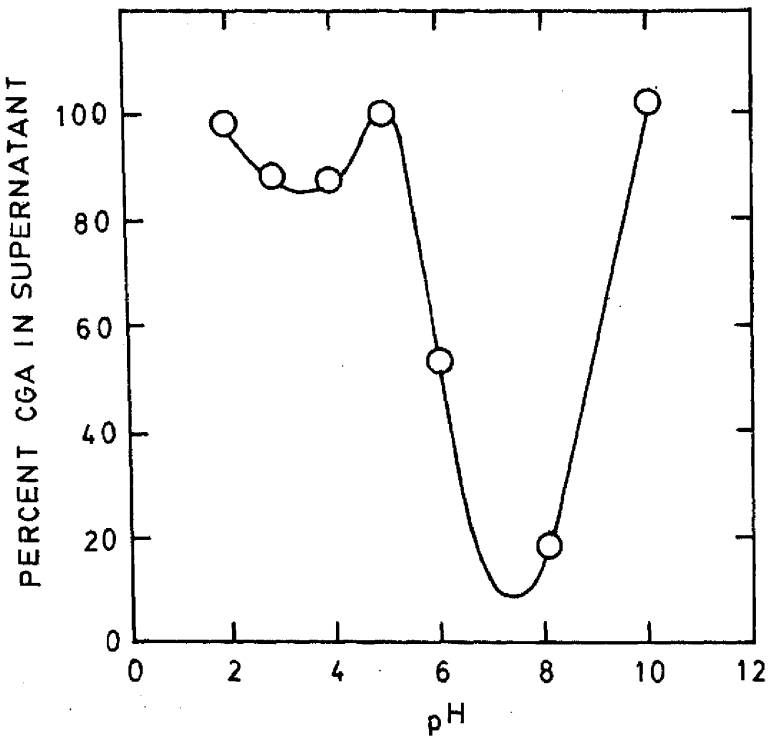

Figure 2. Effect of pH on the per cent CGA in the supernatant of helianthinin at $9{\cdot}6 \times 10^{-5}$ M CGA after 1 h incubation at 27°C.

3.2 *Characterization of the interaction by various techniques*

3.2a *Ultraviolet difference spectroscopy:* Interaction of helianthinin with CGA has been studied by ultraviolet difference spectroscopy and the resultant spectra is shown in figure 3A. It has peaks at 243 and 325 nm with a shoulder at 295 nm. There is a small peak at 310 nm indicating the possibility of involvement of the 4-OH group of phenolic ring of CGA in binding. The difference spectral data is analysed by binding isotherm and Klotz plot (figure 3B). From the data the total number of binding sites are found to be 50 ±5 with an association constant of $1.0 \pm 0{\cdot}1 \times 10^{-4}M^{-1}$. Hill coefficient is 1·3. Experimental curve was best fitted using a K_a of $1{\cdot}1 \times 10^4$ M^{-1} and n of 50. This data may not completely represent the entire binding of CGA as there may be sites other than the chromophoric sites wherein CGA could bind. Thus, this data represents only a part of the binding of CGA to the protein.

3.2b *Fluorescence spectroscopy:* Protein fluorescence is much more sensitive to the binding of ligand than the absorption. Hence, interaction of helianthinin with CGA has been studied in much more detail by monitoring the fluorescence emission spectrum of helianthinin. Upon addition of CGA, the intrinsic fluorescence intensity

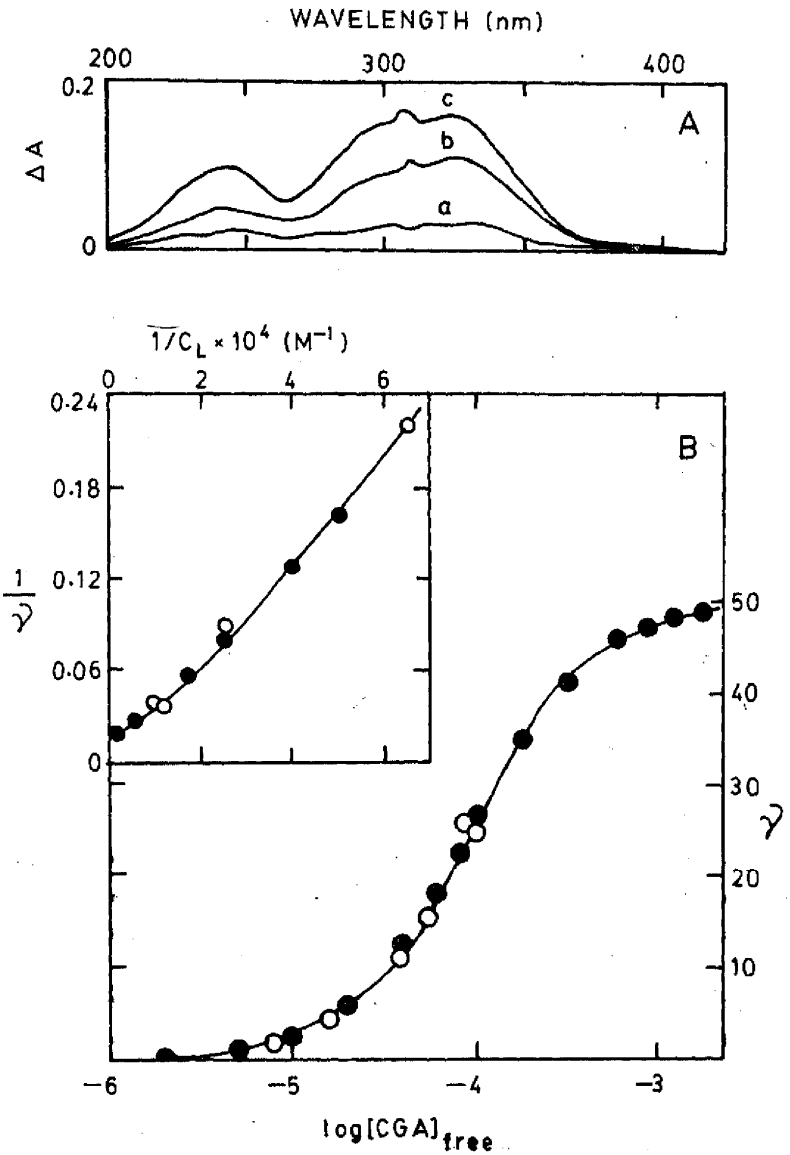

Figure 3. (A) Ultraviolet difference spectra of the helianthinin-CGA interaction at pH 6·0, 25°C in 0·15M PB. The CGA concentrations were **(a)** $2{\cdot}94 \times 10^{-5}$ M, **(b)** $5{\cdot}95 \times 10^{-5}$ M and **(c)** $1{\cdot}89 \times 10^{-4}$ M. **(B)** Binding isotherm of above data analysed at 325 nm. *Inset:* Klotz plot of the binding data. (●), Experimental points; (O), calculated points.

of helianthinin decreases. In figure 4, inset shows the representative inner filter corrected fluorescence emission spectra of helianthinin as a function of increasing CGA concentration. The fluorescence emission spectrum shows a decrease in intensity after giving correction for the inner filter effects. There is a red shift of 5nm in the emission maximum of helianthinin at $1{\cdot}1 \times 10^{-4}$ M CGA at 28°C. The quenching data of the complex is analysed by plotting $\Delta F_{corr}/F_o$ versus CGA concentration

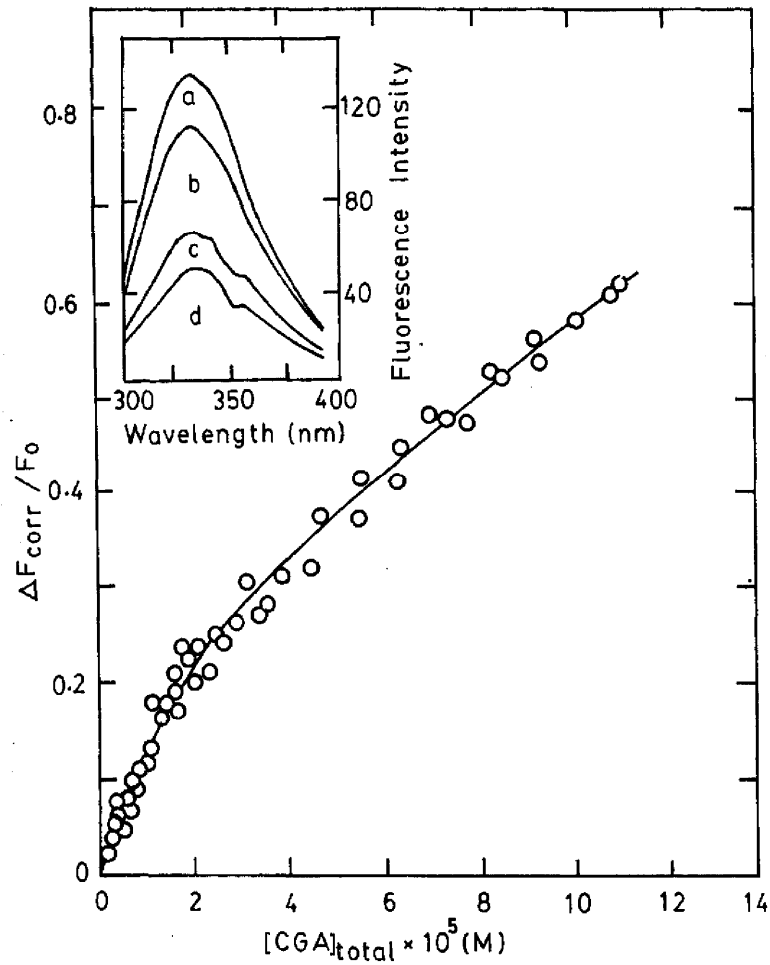

Figure 4. Changes in fluorescence quenching as a result of titration of CGA concentration. *Inset:* Fluorescence emission spectra of helianthinin as a function of CGA concentration, (a), Without CGA; (b), $1{\cdot}12 \times 10^{-5}$ M CGA; (c), $7{\cdot}41 \times 10^{-5}$ M CGA; (d), $1{\cdot}1 \times 10^{-4}$ M CGA. All the spectra have been obtained after giving correction for the inner filter effects.

and is shown in figure 4. The fractional binding plot of the binding of CGA as a function of its free concentration is shown in figure 5. The plot is nearly linear within experimental error over two orders of CGA concentration. The average first association constant was calculated to be $1{\cdot}38 \pm 0{\cdot}1 \times 10^4 M^{-1}$. Total number of binding sites evaluated are 200 ±20.

Binding constant was also evaluated for CGA binding at 28°C in the presence of $3{\cdot}43 \times 10^{-3}$ M Na_2SO_3. The K_a value obtained is $1{\cdot}49 \times 10^4$ M^{-1} which is almost same as that of control value. Na_2SO_3 will provide a sulphite ion which can complex with the basic groups of the protein. It provides sufficient ionic strength more than that of CGA. It also maintains the reducing status of CGA phenolic groups. Therefore evaluation of K_a in the presence of Na_2SO_3 will give an insight

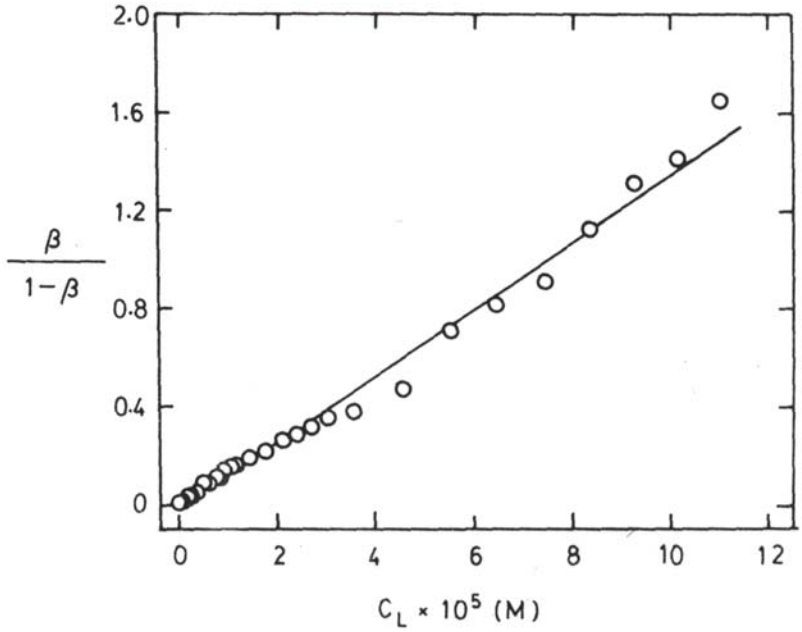

Figure 5. β/1 – β versus free ligand concentrations plot of the interaction of CGA with helianthinin from fluorescence quenching data.

into the (i) minimum structure and forces that are governing the complexation of CGA, (ii) that the binding is noncovalent or covalent and (iii) that the binding is specific or not. The similarity of K_a values in the presence and absence of Na_2SO_3 suggests that not only the chlorogenic acid COO^- group is essential but the entire structure is important in the binding. By ensuring the reducing status of the phenolic groups, the binding that is seen is the noncovalent bonding, but not covalent.

3.2c *Equilibrium dialysis*: Binding isotherm of the CGA binding from equilibrium dialysis experiment is shown in figure 6. The binding isotherm was analysed by Scatchard plot (figure 7A). The concave downward curvature in Scatchard plot shows that there is positive cooperativity in binding. Total number of binding sites were estimated as 400 ±100 from Klotz plot (figure 7B). The Hill coefficient obtained from the graph is 3·67. Based on these initial estimates of the binding parameters, the binding isotherm, Scatchard plot and Klotz plots are simulated by Monte carlo analysis to obtain the best fit line passing through the experimental points. For the best fit, the association constant used was $3{\cdot}5 \pm 0{\cdot}1 \times 10^4$ M^{-1} and number of binding sites used are 420.

3.2d *Sedimentation equilibrium and sedimentation velocity:* Sedimentation equilibrium pattern of helianthinin and helianthinin equilibrated with CGA is shown in figure 8. The control protein has a molecular weight of 2,41,500 ± 12,000. Helianthinin equilibrated with CGA concentration of $7{\cdot}46 \times 10^{-6}$ M sediments with a higher molecular weight of 2,68,000 ± 115,000. This suggests that the number of CGA molecules bound to the protein are enormously high, as already indicated in the binding data, with alteration in the weight average molecular weight of the protein

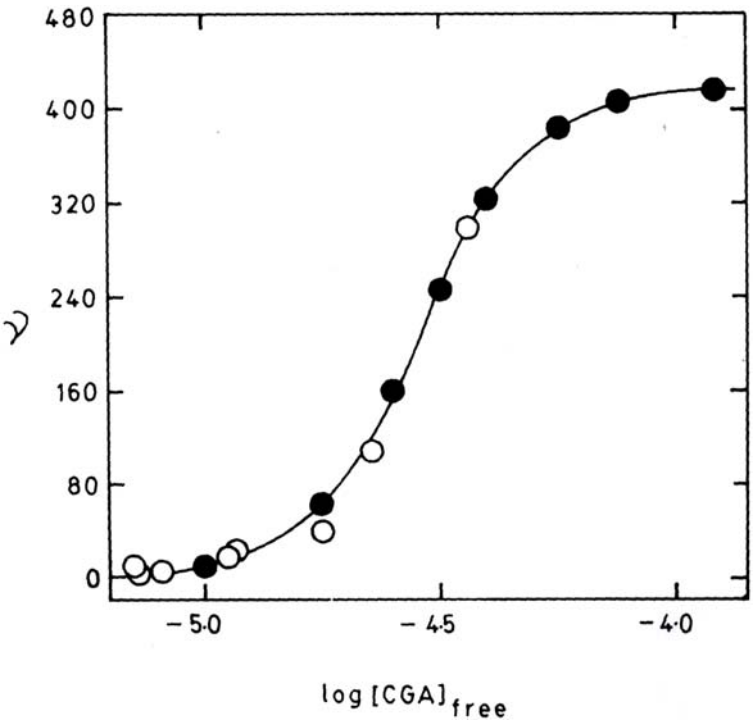

Figure 6. Binding isotherm of the interaction of CGA with helianthinin by equilibrium dialysis. (●) Experimental points; (O), calculated points.

by nearly 30,000. This is supported by the data that an increase in the sedimentation coefficient of helianthinin from 11·1 S to 11·6 S is observed upon binding of CGA. However, there is no change in the association-dissociation property of helianthinin upon complexation.

3.2e *Microcalorimetry*: The calorimetric titration of CGA with helianthinin is performed to obtain the changes in energy as a result of binding of CGA to protein. In figure 9A is shown the raw data of the changes in the reaction cell as a function of time. The above data has been analysed for the heat changes with the injection number using Origin Software provided with the instrument (figure 9B). In figure 9B is seen a break in the curvature of the changes in micro calories. This data suggests that there are at least two classes of binding of CGA to the protein. Possibly the two curves that one can see in figure 9B correspond to high affinity and low affinity sites. This heterogeneity in binding is not observed from other experiments probably because of the overlapping contributions of binding to individual amino acids such that a linear signal is observed as a function of CGA concentration.

3.3 *Structural changes of helianthinin upon binding*

3.3a *Fluorescence*: There was a shift of 5 nm in the fluorescence emission maxima of helianthinin upon complexation with CGA (at a concentration of $1{\cdot}1 \times 10^{-4}$M).

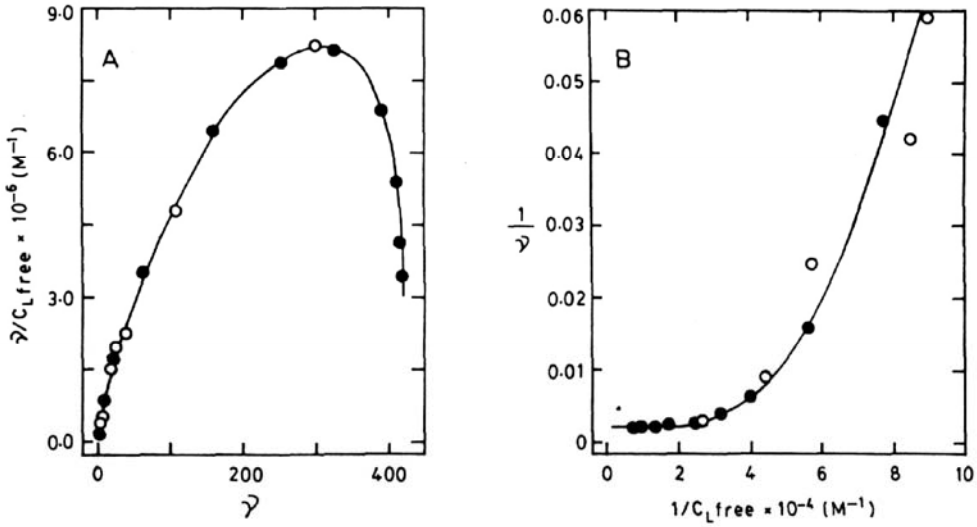

Figure 7. (**A**)Scatchard plot of the binding data obtained from equilibrium dialysis. (**B**) Klotz plot of the interaction data.(●) Experimental points; (O), calculated points.

This is an indication that the tryptophan residues are experiencing a more polar environment.

3.3b *Thermal transition temperature measurement:* The apparent transition temperature (T_m) of the protein is 76°C from first derivative analysis. In the presence of 8·88 × 10^{-5}M CGA the *Tm* decreases by 2°C. At 1 × 10^{-3}M CGA there is nearly 4°C decrease in T_m. The decrease in T_m indicates that complexation destabilizes the protein.

3.3c *Circular dichroic spectra:* Figure 10 shows the circular dichroic spectra of helianthinin in the presence and absence of CGA. Analysis of the data revealed that helianthinin has 9% α-helix, 19·5% β-sheet and 28·5% β-turn content, the rest being aperiodic structure. At 1 × 10^{-5} M CGA the α-helix, β-sheet and β-turn contents of the protein are 7%, 24·5% and 26·5%, respectively. And at 8 × 10^{-5}M CGA the α-helix, β-sheet and β-turn contents of the protein are 6·5%, 23·5% and 26% respectively.

3.4 *Nature and mechanism of interaction*

Since CGA binding perturbs both the ultraviolet absorbance and fluorescence properties, it is expected as a first approximation that aromatic chromophores are involved in the binding. To find out conclusively the involvement of tryptophan and tyrosine residues, further experiments were carried out and the results are discussed as follows.

We have chemically modified the protein with NBS. Chemical modification of helianthinin with NBS shows that there are six exposed tryptophan residues out of

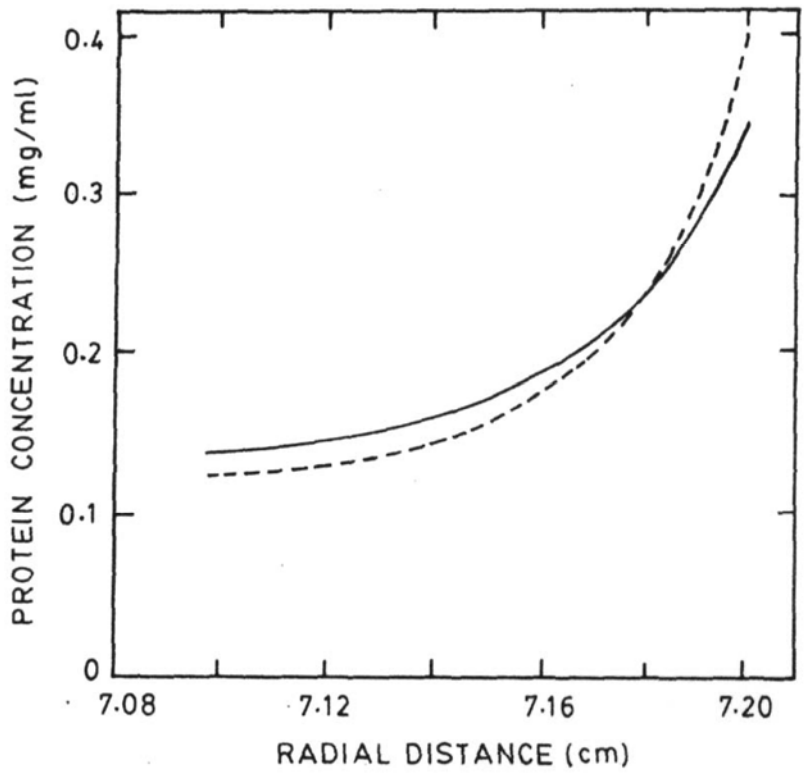

Figure 8. Distribution of protein helianthinin (————)and helianthinin containing $7{\cdot}7 \times 10^{-6}$M CGA (– – – – –) as a function of radial distance in the centrifuge cell in sedimentation equilibrium experiment.

a total of 20 ± 1 residues. Modification of these residues by NBS alters the intrinsic fluorescence emission maximum of the protein. The 332 nm peak decreases in intensity with the appearance of an additional peak at 308 nm. Tyrosine in aqueous media has its emission maximum at 303 nm (Teale and Weber 1957). This suggests that tyrosine dominates the fluorescence of tryptophan modified protein. Addition of CGA quenches the intrinsic fluorescence intensity of the tryptophan modified protein at 308 nm. This indicates that exposed tyrosine groups are interacting with CGA. As can be seen from table 1, there is a decrease in the binding constant of CGA to helianthinin upon NBS modification. The number of binding sites decreased from 200 to 133 ± 15. This suggests that tryptophan modification brings about a major change in the binding of CGA to helianthinin. However, we can not explain with this data the decrease of 67 binding sites though only 6 tryptophan residues were modified.

We have added the CGA to the protein and then NBS titration was carried out. This experiment will tell whether the exposed tryptophan residues are free to be modified or complexed with CGA so that they will be protected from modification. Exposed tryptophan residues are not modified by NBS in the presence of CGA suggesting that there is complete protection of indole moieties towards modification as a result of the binding of CGA.

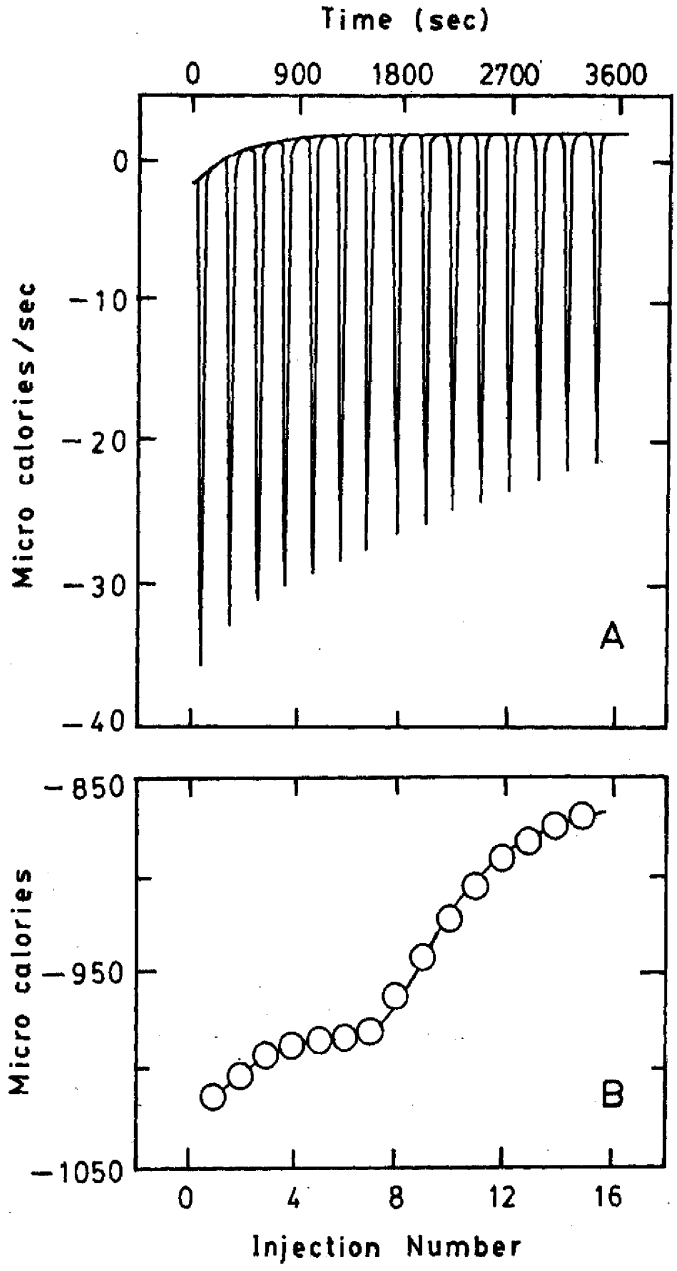

Figure 9. Microcalorimetric data of $7{\cdot}26 \times 10^{-6}$ M protein being titrated versus $2{\cdot}41 \times 10^{-4}$ M CGA. **(A)** Raw data of the energy changes in the titration cell. **(B)** Analysed- and curve fitted data of the titration up to a titration of 15 injections over a period of 3600 s.

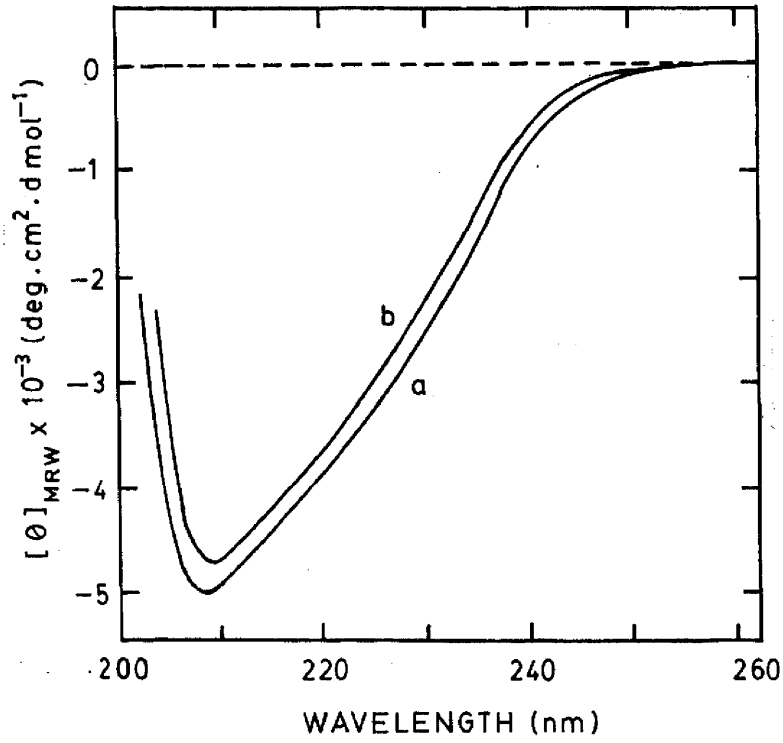

Figure 10. Circular dichroic spectra of (a) helianthinin and (b) helianthinin in the presence of 1×10^{-5} M CGA.

Further, the polarizability of the protein has increased from 0·107 to 0·114 with binding indicating the tryptophan residue participation in the complex formation.

Convincing evidence for the involvement of individual side chains is obtained by a study of the interaction of CGA with free amino acid side chain moieties by fluorescence spectroscopy. CGA binds with N-acetyl L-tryptophan ethyl ester with association constants K_{a1} and K_{a2} of $2{\cdot}3 \pm 0{\cdot}3 \times 10^4$ M $^{-1}$ and $9{\cdot}0 \pm 0{\cdot}5 \times 10^3$ M^{-1} respectively with two molecules of CGA binding to each molecule of the tryptophan moiety. CGA binds with N-acetyl L-tyrosine ethyl ester with an association constant $5{\cdot}7 \times 10^3$ M^{-1} with a stoichiometry of 1:1. These results conclusively shows the involvement of tryptophan and tyrosine residues in the binding process.

Addition of ANS, a hydrophobic probe, to the protein decreased the intrinsic fluorescence intensity of the protein along with an increase in ANS quantum yield at 460 nm. This suggests that tryptophan/tyrosine residues are involved in its binding. ANS binding saturates at $2{\cdot}84 \times 10^{-5}$M. Titration of 11S-ANS complex (11S + $2{\cdot}84 \times 10^{-5}$ M ANS) with CGA led to the association constant of $1{\cdot}33 \times 10^3$ M^{-1} which is a 10-fold decrease in the affinity as compared with the native protein. This result shows that hydrophobic amino acids such as tryptophan, tyrosine and other non-aromatic amino acids are involved in the binding of CGA.

Since hydrophobic residues are the expected choice for the binding sites, the interaction should show major changes in the strength of binding when the temperature is varied. Table 2 shows the n and K_a values as a function of temperature. As

Table 1. Binding parameters of interaction of CGA with lysine modified helianthinin and tryptophan modified helianthinin at 28°C.

Modification	$K_a \times 10^{-4}$ M (M^{-1})	n_{total}
Control helianthinin	1·38 ± 0·20	200 ± 20
Lysine modified helianthinin	0·98 ± 0·05	170 ± 20
Tryptophan modified helianthinin	0·51 ± 0·05	133 ± 15

Table 2. Thermodynamic parameters of the interaction of CGA with helianthinin determined by fluorescence spectroscopy.

Temperature (°C)	$K_a \times 10^{-4}$ M (M^{-1})	n_{total}
10·2	2·10 ± 0·40	140 ± 10
20·0	1·20 ± 0·05	130 ± 10
28·0	1·38 ± 0·1	200 ± 20
35·0	1·60 ± 0·1	170 ± 20
40·2	1·90 ± 0·2	123 ± 10
45·0	2·05 ± 0·2	59 ± 5

temperature increases from 10·2°C to 45°C, the K_a of CGA binding increases non-linearly. The binding constant data was further analysed by van't Hoff plot in the temperature range 20°C and 45°C. The $\Delta H°$ and $\Delta S°$ values were calculated to be 4532 ± 889 cal/mol and 34 ± 3 cal/mol/deg, respectively. The positive values of $\Delta H°$ and $\Delta S°$ shows that the interaction is predominantly due to entropically driven hydrophobic interaction (Hjerten *et al* 1974). However, for the reaction between 10·2°C to 20·0'C, the $\Delta H°$ and $\Delta S°$ values were – 9420 ± 2250 cal/mol and 13·5 ±7·6 cal/mol/deg. These values indicate that ionic interaction/hydrogen bonding is predominant at low temperatures. Spectroscopic results thus conclusively show that both hydrophobic and ionic interactions are present in the binding of CGA. This is supported by *n* which has a total number of 200 by fluorescence spectroscopy (mainly arising out of tryptophan/tyrosine interactions) and 420 number from equilibrium dialysis (arising out of all possible modes of interaction) suggesting that predominantly hydrophobic interaction is responsible for the interaction of CGA with helianthinin.

Since ionic interaction is also found to be present in this complexation, chemical modification of lysyl residues of helianthinin by succinic anhydride was done. Number of lysine residues of native helianthinin are 44 as determined by amino acid composition (Prakash and Rao 1986). The extent of succinylation was 86 ± 2% as determined by the available lysine content. Succinylation shifts the intrinsic fluorescence emission maximum of helianthinin from 332 to 340 nm. This suggests that either conformational changes of protein involving the exposure of tryptophan residues, or interaction of nearby charged groups with tryptophan is occurring upon succinylation. Addition of CGA quenches the intrinsic fluorescence intensity of the succinylated helianthinin. In table 1 the K_a values of binding of CGA to succinylated helianthinin is shown. There is a decrease in the association constant of CGA binding. The number of binding sites are also decreased from 200 to 170 upon

succinylation. The decrease in the number of binding sites upon modification of the exposed lysyl residues of helianthinin suggests the involvement of lysyl residues in the binding process.

From the results described, it is evident that both hydrophobic and ionic interactions are present in the interaction between CGA and helianthinin, a multisubunit protein. The extent to which these individual forces contribute to the total interaction is dependent on the pH, temperature and ionic strength. Such hydrogen bonding/ionic interaction or hydrophobic interaction were envisaged in the interaction of polyphenols with monomeric proteins (Van Buren and Robinson 1969; Loomis 1974; Oh *et al* 1980; Haslam and Lilley 1988). However, combination of these forces are not reported to be involved in binding of polyphenols with proteins. Preliminary work on the nature of the interaction using PAGE and pH stat titration techniques, indicated that ionic interaction is one of the principal force, apart from the hydrophobic interaction, involved in the binding of CGA to helianthinin. Since caffeic acid part of CGA is hydrophobic and quinic acid part has hydrophilic group (see figure 1), it is postulated that both hydrophobic and ionic/hydrogen bonding interactions are present simultaneously in the binding of CGA to helianthinin.

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