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Chlorogenic Acid Interactions with Sunflower Proteins

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The interaction of chlorogenic acid with sunflower (*Helianthus annuus*) protein isolate was investigated with continuous diafiltration at three ligand to protein molar ratios and at four pHs. The pH had a profound influence on the extent of binding. The molar binding ratio was lowest at pH 5, irrespective of ligand to protein molar ratio. Binding was greater at pH 7 and pH 3. At pH 9, binding was lower than at pH 3 up to a certain free chlorogenic acid concentration. Above this concentration, binding at pH 9 was higher than at other pHs. Binding increased as the ligand to protein molar ratio increased irrespective of pH. There are two groups of binding sites in sunflower proteins at pH 3, 5, and 7 and three groups at pH 9.

Polyphenolic compounds have been a major deterrent to large-scale use of sunflower proteins in food products, due to their chromophoric properties. If the pH of sunflower flour or meal, which contains 3-5% by weight phenolic compounds, is raised above neutrality, its color progresses from a cream yellow to light green, to dark green, and finally to brown. It is desirable for vegetable proteins to be odorless, bland, and colorless. Many of the

current methods for producing such protein isolates either mask the color changes or add extra processing steps that increase the cost. Understanding the reaction mechanisms can lead to innovative processes to develop such products.

Phenolic compounds in sunflower products include chlorogenic acid, caffeic acid, and quinic acid (Joubert, 1955; Sechet-Sirat et al., 1959), varying with the location of the seed on the sunflower head, storage temperature (Pomenta and Burns, 1971), and variety (Sosulki et al., 1972). Chlorogenic acid (3-*O*-caffeoyl-D-quinic acid) is the major phenolic compound. Acid, base, or enzymatic hydrolysis of the ester linkage of chlorogenic acid yields caffeic acid and D-quinic acid. True chlorogenic acid is

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only one of several positional isomers. The other chlorogenic acid isomers, called iosochlorogenic, pseudochlorogenic, and neochlorogenic acid, differ in the point of attachment of the caffeic ester linkage to the quinic acid ring.

During processing, the cells in sunflower seeds may be ruptured, releasing polyphenoloxidase, which catalyzes the oxidation of polyphenols to o-quinones (Pierpoint, 1969). The o-quinones are highly reactive and may bind covalently with thiol or amino groups of proteins. Since sunflower proteins are already deficient in lysine, this interaction further lowers the nutritive value in as much the new condensation products cannot be metabolized by humans (Loomis, 1974; Synge, 1975). Polyphenolic compounds may also react noncovalently with protein via hydrogen-bonding, ionic, and hydrophobic interactions.

In order to develop methods of separating or removing these compounds from sunflower protein products, a more thorough understanding of the nature and extent of the binding of these phenolic compounds is needed. This paper reports on a study of the binding of chlorogenic acid to a sunflower protein isolate by the continuous ultrafiltration (diafiltration) method to obtain binding parameters. The main advantage of this method is its rapidity. The effect of free species concentration on the binding equilibrium, and an entire binding isotherm, could be determined with a single run of no more than a few hours duration (Cheryan and Saeed, 1989).

MATERIALS AND METHODS

Preparation of Sunflower Protein Isolate. The method of Saeed and Cheryan (1988) was used to prepare a low-polyphenol, reduced-phytate protein isolate (LPRP) from defatted sunflower meal. The composition of LPRP (percent dry basis) was as follows: lipids, 0.23; protein (Kjeldahl N × 6.25), 94.1; ash, 1.4; phytic acid, 0.35; polyphenols, <0.05%. Standard methods (AOAC, 1984) were used for the analysis. Polyphenolic compounds were determined by the method of Dorrell (1976), and phytic acid was determined as described earlier (Saeed and Cheryan, 1988).

Binding Studies. The "continuous ultrafiltration/diafiltration" technique has been described by Cheryan and Saeed (1989). Binding studies of chlorogenic acid to sunflower protein isolates was done under the following conditions: stirring speed, 200–300 rpm; temperature, 22 ± 1 °C; membrane, YM-10 (Amicon Corp., Danvers, MA); buffer, 0.15 M NaCl. The independent variables and their levels are shown:

variable	levels
protein concn, % w/v	0.2, 0.5
chlorogenic acid concn, mM	0.10, 0.15
ligand to protein molar ratio	2, 5, 7.5
pH	3, 5, 7, 9

The protein and ligand solutions were made up in 0.15 M NaCl. Sample size in the stirred UF cell was 50 mL. For each experiment, the pHs of the protein solution and the ligand solution were preadjusted to the required pH with 0.5 N HCl or 0.5 N NaOH. Nitrogen at 20 psig was applied to the system through the selector valve as the transmembrane pressure.

The permeate was collected in 5-mL fractions in the fraction collector. The ligand–solute interchange (i.e., the diafiltration) was allowed to continue under constant conditions until about five volumes had permeated through the cell. This means that if the cell contents were 50 mL, 250 mL of permeate was collected in 5-mL aliquotes, for a total of 50 aliquots. This would give us a V_D of 5, where

$$V_D = \text{vol permeated/vol in UF cell}$$

It can be theoretically shown that a V_D of 5 will result in more than 99% exchange of the cell contents with the ligand solution (Cheryan, 1986; Cheryan and Saeed, 1989). The permeate samples were analyzed for chlorogenic acid and/or protein as needed.

Assay of the Permeate Samples. *Chlorogenic Acid.* A solution of chlorogenic acid (Sigma Chemical Co., St. Louis, MO)

in 0.15 M NaCl has an absorption maximum at a wavelength of 328 nm at pH 3, 5, and 7. However, at pH 9 the absorption maximum is 376 nm. The absorptivity of chlorogenic acid solution was found to vary linearly with the concentration of the acid between 0.01 and 0.15 mM at the wavelength of maximum absorption. Standard curves of chlorogenic acid solution in 0.15 M NaCl were prepared at each pH. The molar absorptivities of chlorogenic acid are as follows: pH 3, 1.853 × 10⁴; pH 5, 1.796 × 10⁴; pH 7, 1.538 × 10⁴; pH 9, 1.010 × 10⁴.

The concentration of chlorogenic acid in the permeate samples was determined by measuring the absorptivity at the maximum wavelength at that pH and converting the value into millimolar units of chlorogenic acid by the standard curves.

Protein. The Biuret test (Gornall et al., 1949) was used for measuring protein in the cell solution but was not suitable for measuring low protein levels expected in the permeate. A spectrophotometric method (measuring the absorbance of the solution at 280 nm) was found to be a simpler and more direct method for measuring the protein content of permeate. Samples were diluted 1:10 and then read directly in a silica rectangular cell (10 mm) on a recording spectrophotometric (Shimadzu Corp., Kyoto, Japan). A standard curve of protein concentration versus absorbance at 280 nm with sunflower protein isolate was prepared.

Calculations of Binding Parameters. The procedures for converting the raw data to binding parameters using a personal computer spreadsheet program (Lotus 1-2-3) has been given by Cheryan and Saeed (1989). For calculation purposes, the molecular weight of the LPRP sunflower protein isolate was assumed to be 100 000 based on the data of Kabirullah and Wills (1983).

RESULTS

Membrane Rejection Characteristics. As emphasized by Cheryan and Saeed (1989), a critically important factor when membrane methods are used to study binding is to account for nonspecific interactions between the membrane and the ligand or the protein and, more important, to select a membrane that retains the proteins completely but allows free passage of the ligand. Most commercial membranes are unfortunately not ideal in that they do not possess the desired sharp molecular weight cutoff (Cheryan, 1986). Thus, one must account for the possible retention of the ligand and the permeation of the protein. Of all the membranes tested in our laboratory, the Amicon YM-10 membrane had the lowest nonspecific protein binding. This membrane was used in all our binding studies. Protein rejection in the first fraction through the membrane was 98.1–98.6%, but in all subsequent fractions, rejections were above 99% at all pHs (Saeed, 1987).

The rejection coefficient of chlorogenic acid when dissolved in water was 10–40%. Since chlorogenic acid has a molecular weight of 354.6 and the YM-10 membrane has a molecular weight cutoff of 10 000, these high rejections indicate that chlorogenic acid could be polymerizing or binding to the membrane. The rejections were significantly reduced to 0–5% when 0.15 M NaCl was used as the solvent (Saeed, 1987). All binding studies were thus conducted with both protein and chlorogenic acid in 0.15 M NaCl.

Binding Curves and Parameters. Binding curves showing the molar binding ratio (r, moles of chlorogenic acid per mole of protein) vs free chlorogenic acid concentration (A_f) as a function of pH are given in Figures 1–3. The corresponding Scatchard plots are shown in Figures 4–7. The Scatchard model is based on the equilibrium between a ligand and the protein and is commonly expressed as (Cheryan and Saeed, 1989)

$$r = \frac{nK_aA_f}{1 + K_aA_f}$$

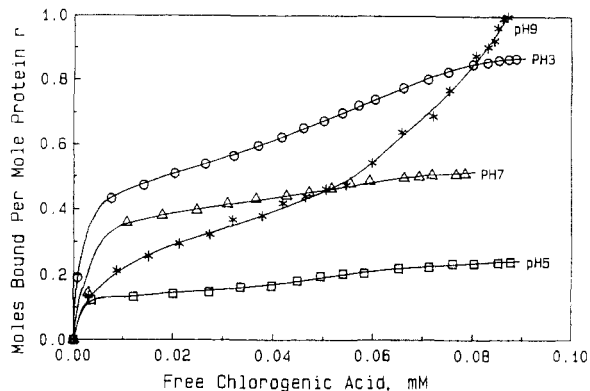where n is the number of independent binding sites, K_a

Figure 1. Binding of chlorogenic acid to sunflower proteins as a function of pH. Molar ratio of ligand to protein is 2.
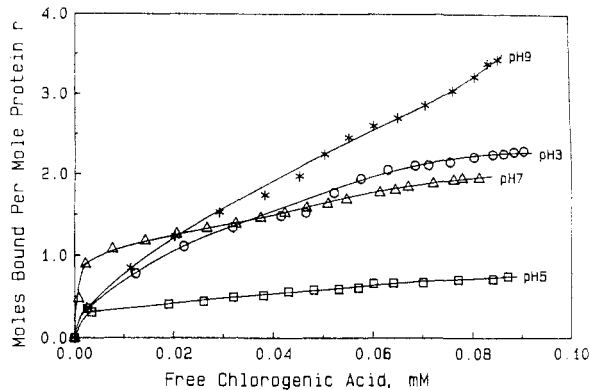

Figure 2. Binding of chlorogenic acid to sunflower proteins as a function of pH. Molar ratio of ligand to protein is 5.
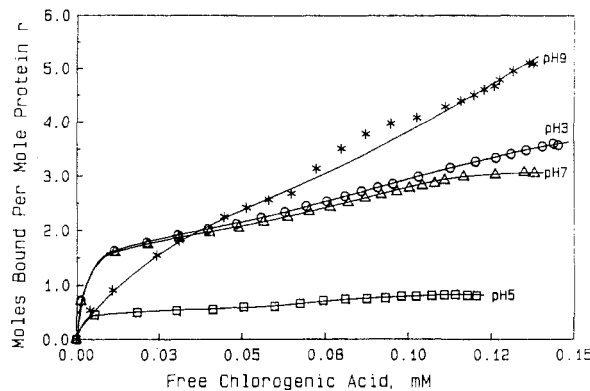

Figure 3. Binding of chlorogenic acid to sunflower proteins as a function of pH. Molar ratio of ligand to protein is 7.5.

Figure 4. Scatchard plot of chlorogenic acid–sunflower protein binding as a function of ligand to protein molar ratio, pH 3.

Figure 5. Scatchard plot of chlorogenic acid–sunflower protein binding as a function of ligand to protein molar ratio, pH 5.

Figure 6. Scatchard plot of chlorogenic acid–sunflower protein binding as a function of ligand to protein molar ratio, pH 7.

Figure 7. Scatchard plot of chlorogenic acid–sunflower protein binding as a function of ligand to protein molar ratio, pH 9.

is the association constant, and A_f is the concentration of free ligand in the cell. This can be written in the form

$$r/A_f = K_a n - K_a r$$

For a set of equivalent binding sites, these plots would be straight lines, with the intercept on the abscissa giving n and the slope giving K_a. Since the plots are nonlinear, the binding apparently involves more than one type of binding site. In such cases, one commonly used method for obtaining values of n is to fit straight lines to the linear portions of the data. The Lotus 1-2-3 spreadsheet program we used for data handling contains a linear regression subroutine that allows us to select the range of values that we wish to linearize. The range of points was selected on the basis of the best correlation coefficient. Table I lists the values of n and K_a obtained in this study.

The number of binding sites increased as the molar ratio of ligand to protein increased from 2 to 7.5 at all pHs. Minimum binding occurred at pH 5 at all ligand to protein
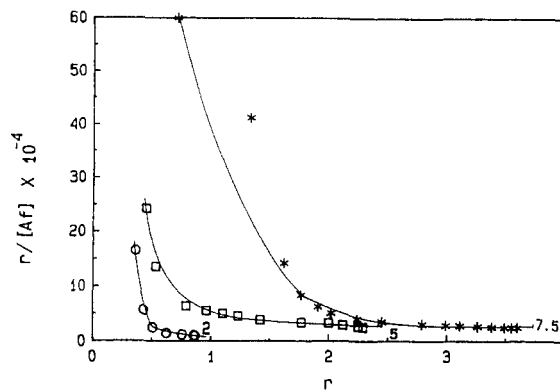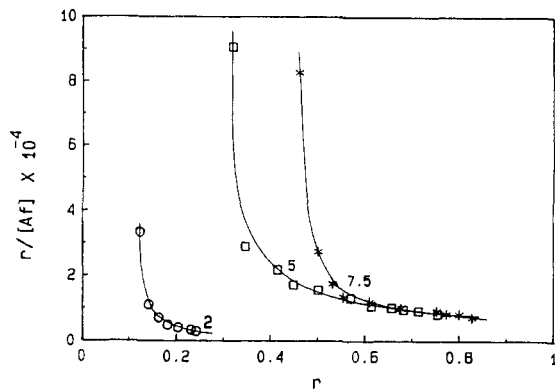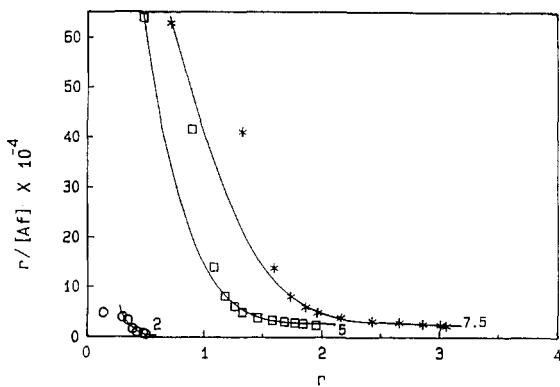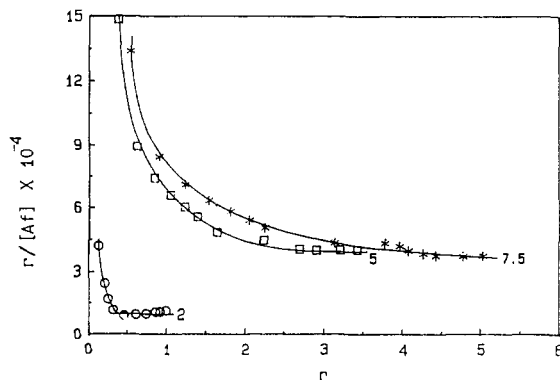
ratios. Binding at pH 3 and 7 was much higher than at pH 5. At pH 9, the initial binding was lower than pH 3,

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Table I. Binding Parameters of Chlorogenic Acid with Sunflower Proteins

molar ratio[a]	pH	n_1	n_2	n_3	$10^5 K_1$, M^{-1}	$10^4 K_2$, M^{-1}	$10^3 K_3$, M^{-1}
2	3	0.53	0.66		7.57	2.94	
	5	0.14	0.22		1.39	2.40	
	7	0.42	0.23		6.63	5.21	
	9	0.38	1.15		1.65	1.38	
5	3	1.11	2.61		1.72	1.80	
	5	0.44	0.67		6.07	2.41	
	7	1.31	1.47		7.98	2.91	
	9	1.28	1.84	10.0	1.57	3.24	3.99
7.5	3	1.98	4.43		5.04	8.71	
	5	0.56	0.64		7.36	2.01	
	7	1.94	2.57		5.31	1.61	
	9	1.97	2.86	9.8	1.77	1.96	3.83

[a] Ligand to protein molar ratio, assuming molecular weight of protein is 100 000.

Table II. Maximum Molar Binding Ratios (Moles of Chlorogenic Acid Bound per Mole of Protein)

ligand to protein molar ratio	pH 3	pH 5	pH 7	pH 9
2	0.86	0.24	0.57	0.99
5	2.29	0.75	1.95	3.43
7.5	3.59	0.83	3.06	5.10

but at higher ligand concentrations, the binding was higher than at any other pH (Figures 1–3). There are no consistent trends with the association constants, which vary from (1.39–7.98) × 10⁵ M⁻¹ for K_1 to (3.83–3.99) × 10³ M⁻¹ for K_3. Our studies suggest that sunflower protein has two groups of nonidentical binding sites at the three lower pHs and three binding sites at pH 9.

Table II lists the maximum molar binding ratios obtained in this study. The data indicate that 0.1 mM chlorogenic acid (i.e., at an initial molar ratio of 2) was not enough to saturate all the binding sites of 0.5% protein. It is obvious that the concentration of chlorogenic acid relative to the concentration of protein is important and should be such as to cause the complete saturation of all the binding sites of the protein.

DISCUSSION

Not surprisingly, minimum binding was observed at pH 5.0, which is the isoelectric point of sunflower proteins. This could be due simply to the insolubility of the protein, which makes the binding sites less available to the ligand. It may also indicate that electrostatic type interactions are minimum, although hydrophobic association between chlorogenic acid and sunflower protein is possible. Chlorogenic acid is an amphiphile with a hydrophobic phenyl ring and a carboxylic ring of a more hydrophilic nature at opposite ends of the molecule. Such molecules are known to have an affinity for exposed hydrophobic residues on proteins (Tanford, 1980; Damodaran and Kinsella, 1980). Hence, the possibility exists that hydrophobic and van der Waals forces are also important in phenolic–protein interactions and could account for the low amount of binding observed at pH 5.0.

Low pH. Interaction of chlorogenic acid with sunflower proteins was much higher at pH 3.0. Binding at low pH is generally attributed to hydrogen-bond formation between phenolics and proteins (Sabir et al., 1974), involving unionized hydroxyl groups of phenolic compounds and the carbonyl oxygen in the peptide bond of protein. The best evidence for this mechanism comes from studies with collagen. Green (1953) found that lysine and hydroxylysine residues of collagen are important for collagen binding of tannic acid and benzoquinone. Neither the terminal amino nor terminal carboxyl groups played a significant role in

the binding of these tanning agents. The interactions of mimosa tannins with collagen have also been studied (Gustavson, 1954). It was observed that the degree of protein swelling governed the number of carbonyl oxygen atoms of peptide bonds being available for hydrogen bonding. A distinct optimum pH for gelatin–tannic acid precipitation was observed at pH 4.7–4.8, slightly below the isoelectric point (pH 5.2) of calfskin gelatin. Apparently, the peptide groups in gelatin are directed outward, approximately perpendicular to the polypeptide chain. Very stable hydrogen bonds may form especially with polyhydroxy phenolic compounds that are bound at more than one site.

High pH. The binding of chlorogenic acid to sunflower proteins at alkaline pH may be attributed to electrostatic interaction. Chlorogenic acid has a carboxylic acid group that is predominantly ionized at neutral pH. Thus, electrostatic interactions between chlorogenic acid and the still positively charged side chain groups of the protein (e.g., ε-NH₂ of lysine) can take place. Only indirect evidence for the existence of electrostatic phenolic–protein interactions is available in the literature. Sunflower proteins are normally extracted with use of neutral salt solutions (2.5–10% NaCl). Salt extraction no doubt increases the solubility of sunflower seed globulins but was also found to decrease the amount of chlorogenic acid bound to the protein (Sabir et al., 1974). Lahiry et al. (1977) removed 69% of chlorogenic acid from leaf protein concentrate (LPC) using a buffer containing 2.5% sodium chloride, 1 mM mercaptoethanol, and 40 mM sodium phosphate (pH 7.0). As the phosphate concentration in the extraction buffer was increased from 10 to 40 mM, the removal of bound chlorogenic acid from LPC also increased. In his method for crystallization of tobacco fraction 1 proteins, Lowe (1977) used an extremely high salt concentration (5 M) in the grinding buffer. The high sodium chloride concentration effectively inhibited polyphenoloxidase activity but was also thought to obviate electrostatic phenol–protein interactions.

The binding of chlorogenic acid to sunflower protein at pH 9 was lower than pH 3 up to a certain free chlorogenic acid concentration. Above this concentration, the binding at pH 9 was higher than at other pHs. In fact, what appeared to be saturation binding was observed only at a ligand to protein molar ratio of 7.5. The pK of the phenolic OH groups of chlorogenic acid are approximately pH 9.0. The ionized phenolic groups could undergo autoxidation via semiquinone radical(s) to form o-quinones. Because of delocalization of electrons on the phenyl group, this o-quinone form of chlorogenic acid is susceptible to nucleophilic attack by free thiol or amino groups of the protein. Covalently cross-linked tannin–protein complexes are believed to be formed in this manner.

Proteins possess several functional groups in amino acid side chains that can donate a pair of electrons to a quinone molecule. o-Quinones are particularly susceptible to nucleophilic attack at the 4- and 5-positions on the benzene ring (Mason, 1955). Upon nucleophilic attack of a quinone by a side-chain group on a protein molecule, a covalent bond is formed between the protein side chain group and the o-quinone. As a result, the quinone is reduced to its phenolic form. Free sulfhydryl groups in proteins are strong nucleophiles that may react covalently with o-quinones in this manner. Amino groups in proteins can also react covalently with quinones (Mason, 1955; Pierpont, 1966).

It is clear that various mechanisms can be postulated to explain the nature and extent of the binding phenom-

enon that we observed. However, to further elucidate the nature of the binding, several additional experiments need to be conducted. For example, binding studies in the presence of urea will establish whether hydrogen bonding or covalent bonding at pH 9 is predominant. Binding studies in the presence of a chaotropic salt such as sodium thiocynate at pH 5 will confirm whether hydrophobic association is taking place at this pH. Binding studies carried out at increasing ionic strength at pH 7 will establish whether ionic association is the predominant force at this pH. In addition, protein–protein interactions, degree of denaturation, and polypeptide disruption are other factors that can affect the nature and extent of binding. These experiments will lead to better understanding of the interactions occurring in sunflower proteins.

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