Factors Affecting the Binding of Chlorogenic Acid to Fraction 1 Leaf Protein

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By use of equilibrium dialysis, the binding of chlorogenic acid to fraction 1 protein from spinach was quantified. Scatchard plots exhibited negative cooperativity at 25 °C. Chlorogenic acid binding increased with temperature and above 30 °C was associated by a conformational change(s) in fraction 1 protein. Binding varied little with pH, but a significant increase in chlorogenic acid binding was observed at pH 9.0. Binding decreased with increasing ionic strength when either sodium chloride or sodium thiocyanate was used. Sodium thiocyanate was more effective in reducing binding. The addition of polyphenol oxidase to the model system enhanced binding, but in the presence of 11 mM metabisulfite or 10 mM cysteine binding was reduced to levels approximating that occurring in the absence of enzyme; 5 mM ascorbic acid was less effective. A sequential two-step equilibrium dialysis method indicated that chlorogenic acid associated with fraction 1 by both covalent and noncovalent interactions.

Polyphenolic compounds are abundant in foods of plant origin. Their presence is partly responsible for the characteristic color, flavor, and astringency of beverages like tea, cider, and beer and fruits such as cranberries, plums and grapes (Maga, 1978). Small, but significant, quantities of polyphenols are found in oilseeds, especially sunflower and cottonseed (Sosuluski, 1979) and cereal grains, sorghum (Nelson and Cummins, 1975). Polyphenolic compounds also comprise as much as 5% of the dry weight of green leaves (Andersen et al., 1972). Several researchers have investigated methods for isolating and fractionating the proteins from green leaves (Wang and Kinsella, 1976; Betschart and Kinsella, 1973; Pirie, 1971; Edwards et al., 1975). Few of these investigations have addressed the practical problem of polyphenols associated with leaf proteins. During isolation of leaf proteins, plant cells are ruptured to release leaf tissue juices containing polyphenols. Polyphenolic compounds when acted upon by polyphenol oxidases, present in leaves, may be oxidized to o-quinones (Pierpont, 1969). The o-quinones are highly reactive, and as a singular or polymerized molecule they may bind covalently with thiol and amino groups of proteins (Loomis, 1974; Synge, 1975). Polyphenolic compounds may also react noncovalently with proteins via hydrogen bonding, ionic, and hydrophobic interactions (Loomis, 1974).

The presence of polyphenolic compounds can conceivably affect the quality of leaf protein in several ways: i.e., reduce the digestibility; alter the organoleptic properties (off-color, bitterness, astringency); prolong or shorten storage life and stability; adversely change the functional properties and behavior of leaf protein in food systems. In order to minimize these problems and devise suitable methods for leaf protein isolation, more fundamental knowledge concerning the interactions of polyphenolic compounds with leaf proteins is desirable. Therefore, we investigated the interaction of a representative polyphenolic compound, chlorogenic acid, with fraction 1 leaf protein in a model system.

MATERIALS AND METHODS

Chlorogenic acid was purchased Sigma (St. Louis, MO). All other chemicals used in the study were reagent grade. Distilled and deionized water was used in all experiments.

Protein Isolation. Fraction 1 protein was isolated from spinach leaves by using the method of Jones and Mangan

(1976). Contaminant polyphenolic compounds were separated from fraction 1 protein primarily by Sephadex G-75 chromatography. Chromatography was carried out by using a specially designed Pyrex glass cylinder with a bed volume of approximately 3000 mL equilibrated with 0.6 M, pH 7.0, phosphate buffer containing 0.02% sodium azide. The protein eluted from the column was dialyzed against deionized water for 24 h at 4 °C, lyophilized, and stored at -4 °C.

Electrophoresis. The homogeneity of fraction 1 protein isolated by the method of Jones and Mangan (1976) was checked by using polyacrylamide gel electrophoresis (Figure 1) with purified ribulosebisphosphate carboxylase/oxygenase (EC 4.1.1.39) purchased from Sigma Chemical Co. as a reference standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on slab gels consisting of linear gradients of 5–20% acrylamide. Protein samples 0.1–0.2% were dissolved in 10 mM Tris-HCl dissociating buffer, pH 6.8, containing 8 M urea, 2% SDS, 5 mM DTT, and 0.025% bromophenol blue. Protein samples were loaded at 20 μl/channel and run at 14-mA constant current for about 4 h. Gels were fixed and destained in 20:10:70 methanol–acetic acid–water and stained in the same fixer with 0.05% Coomassie blue R.

Equilibrium Dialysis. Equilibrium dialysis studies were performed using acrylic dialysis cells and dialysis membranes with a 6000 M_r cutoff (Technilab Instruments, Pequannock, NJ). Experimental cells with various concentrations of chlorogenic acid dialyzed against fraction 1 protein were matched with control cells containing the same concentrations of chlorogenic acid dialyzed against a buffer solution. In each experimental cell 4.0 mL of a given chlorogenic acid concentration was added to one compartment and 4.0 mL of a fraction 1 protein, 0.5% solution in buffer, to the other compartment separated by a dialysis membrane. Control cells contained 4.0 mL of a chlorogenic acid solution in one compartment and 4.0 mL of buffer in the other. Preliminary studies revealed that at least 12 h was needed for chlorogenic acid to reach equilibrium. In the described experiments dialysis cells were incubated for 24 h in a New Brunswick controlled-environment shaker.

After reaching equilibrium the cells were removed, and the chlorogenic acid concentration in the compartment to which chlorogenic acid was initially added was determined by using Haephner's reagent (Pomenta and Burns, 1971). The absorbance of the red-violet solutions were measured at 520 nm on a Spectronic 700 (Bausch & Lomb), and the concentration of chlorogenic acid was determined from a

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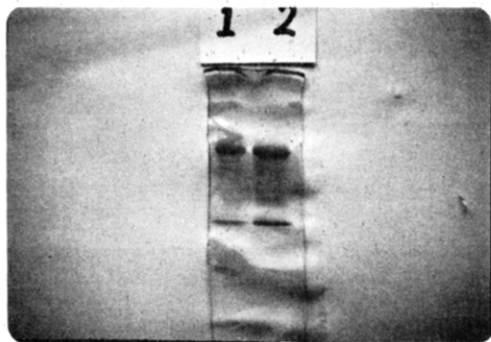
Figure 1. SDS–PAGE gel electrophoretic patterns of fraction 1 protein from spinach. 1 = fraction 1 protein isolated by the method of Jones and Mangan (1976). 2 = ribulose bisphosphate carboxylase/oxygenase.

standard curve. The difference between the chlorogenic acid concentration in the control and experimental cells was attributed to binding of chlorogenic acid to fraction 1 protein. The effects of temperature, pH, ionic strength, reducing agents, and polyphenol oxidase activity on the binding of chlorogenic acid to fraction 1 protein were systematically determined by using this method.

Temperature. The effect of temperature on the binding of chlorogenic acid to fraction 1 was studied by using sets of cells (control and experimental) incubated for 24 h at 10, 25, and 40 °C, respectively. All solutions contained 0.02 M phosphate buffer, pH 7.0, with 0.02% sodium azide to retard microbial growth.

pH. To determine the effects of pH on the binding of chlorogenic acid to fraction 1, equilibrium dialysis experiments were performed at pH 3.0, 5.0, 7.0, and 9.0. All solutions contained 0.15 M NaCl with 0.02% sodium azide and were adjusted to the desired pH with 0.2 N HCl or 0.2 N NaOH. Control and experimental cells were incubated at 25 °C for 24 h before being analyzed.

Ionic Strength. In order to determine if electrostatic interactions between chlorogenic acid and fraction 1 protein are important, equilibrium dialysis experiments were performed at 0.1 and 0.5 M using both NaCl and NaSCN. Sodium chloride is a common electrolyte used in food systems. Sodium thiocyanate is a so-called chaotropic salt because it perturbs the normal hydrogen-bond structure of water and disrupts hydrophobic protein–ligand interactions (Hatefi and Hanstein, 1969). In these experiments dialysis cells were agitated for 24 h at 25 °C.

Reducing Agents and Polyphenol Oxidase. Polyphenol oxidases, enzymes responsible for the enzymatic browning in plant products, are extremely active in leaf tissue juice homogenates. Therefore, we studied the effects of a polyphenol oxidase (PPO) and some PPO inhibitors or reducing agents on the binding of chlorogenic acid to fraction 1 protein using the equilibrium dialysis method. In these experiments all solutions contained 0.02 M, pH 6.5, phosphate buffer with 0.02% sodium azide. Tyrosinase (EC 1.14.18.1, equivalent to a polyphenol oxidase activity of 50 000–100 000 units/mg, Sigma Chemical Co.) was added to the compartment containing fraction 1 protein to give a 1:100 tyrosinase to chlorogenic acid concentration. To assess the effects of PPO inhibitors both control and experiment cells with varying concentrations of chlorogenic acid were run with each of the following inhibitors or reducing agents dissolved in the buffer: 11 mM potassium metabisulfite, 5 mM L-ascorbic acid, and 10 mM L-cysteine. All equilibrium dialysis cells were incubated for 24 h at 40 °C.

**Treatment of the Data.** The difference in the concentration of chlorogenic acid between control and ex-

perimental cells containing fraction 1 protein (after reaching equilibrium) represented the amount of ligand bound to protein. Assuming a molecular weight of 500 000 for fraction 1 protein, the number of moles of chlorogenic acid bound per mole of protein was calculated. Chlorogenic acid in the experimental cells, after reaching equilibrium, was taken as the free ligand concentration, [L]. When $\bar{\nu}$, the number of moles of ligand bound per mole of protein, was plotted against $\bar{\nu}/[L]$, where [L] is the free ligand concentration, a Scatchard Plot of the initial binding data at 25 °C was constructed. The data from the other experiments were analyzed in the form of binding isotherms by plotting $\bar{\nu}$ vs. [L].

Fluorescent Spectra. In order to determine if temperature-induced conformational changes in fraction 1 protein might affect chlorogenic acid binding, fluorescent spectra of both fraction 1 and bovine serum albumin (BSA) were recorded at various temperatures. The excitation and emission wavelengths were 288 and 350 nm for BSA and 287 and 340 nm for fraction 1 protein, respectively. The fluorescent spectra of both proteins were taken at 10-deg temperature intervals from 10 to 50 °C by using a Perkin-Elmer 650-40 fluorescent spectrometer. BSA does not undergo any major conformational changes in this temperature range and thus was used as a control. The sample compartment was gently flushed with nitrogen gas to avoid condensation on the walls of the sample cuvette while the spectra were recorded at 10 °C. The concentrations of BSA and fraction 1 were 0.1% in 0.02 M, pH 7.0, phosphate buffer.

Estimation of Covalent Binding. Since none of the previous experiments were designed to determine if chlorogenic acid is covalently bound to fraction 1 protein, we estimated covalently bound chlorogenic acid using urea to break both hydrogen bonds and dissociate possible hydrophobic interactions between chlorogenic acid and fraction 1. A sequential two-step equilibrium dialysis method was used to estimate the percentage of chlorogenic acid covalently bound. After initial equilibrium dialysis of 4 mM chlorogenic acid against a solution of 0.5% fraction 1 protein in phosphate buffer, the total amount of ligand bound to the protein was quantified by the method previously described. Then the fraction 1 solution containing bound and free chlorogenic acid was removed from the cell and redialyzed against 10 M urea and 4 mM DTT to maintain a reducing environment. A second equilibrium dialysis cell with chlorogenic acid dialyzed against 10 M urea and 4 mM DTT acted as a control. The chlorogenic acid concentration in each control cell was made equal to the total amount (free plus bound) previously determined for the fraction 1 protein solution. After the second dialysis step, aliquots (1 mL) of the urea solution containing chlorogenic acid were removed from both the control and experimental cells. The solutions were diluted 1:5 with 0.02 M, pH 7.0, phosphate buffer, and the chlorogenic acid concentration in each solution was determined by using Haephner's reagent. Absorbance readings were compared with a standard curve of chlorogenic acid in the same buffer containing 1 M urea because higher urea concentrations affected chromophore formation and absorbance at 520 nm. The percentage of chlorogenic acid covalently bound to the protein was then estimated as

% chlorogenic acid covalently bound =
1 − concentration of chlorogenic acid in urea solution (experimental cell)/concentration of chlorogenic acid in urea solution (control cell) × 100

An estimate of the percentage of chlorogenic acid cova-
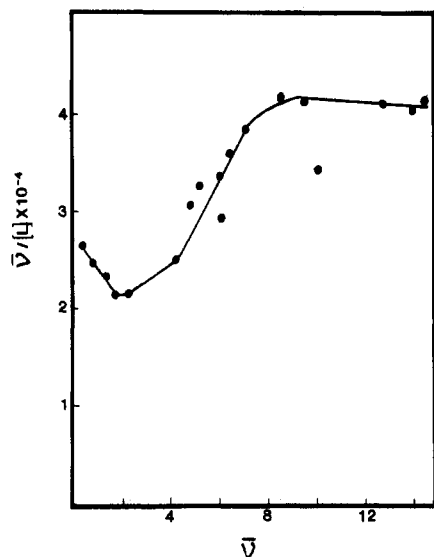
Figure 2. Scatchard plot of the binding of chlorogenic acid to fraction 1 protein at 25 °C. Protein concentration was 0.5% in 0.02 M, pH 7.0, sodium phosphate buffer containing 0.02% sodium azide. $\bar{v}$ represents the number of moles of ligand bound per mole of protein and [L] is the molar concentration of the free ligand.

lently bound to fraction 1 protein under the various experimental conditions was made by using this sequential two-step equilibrium dialysis method.

RESULTS AND DISCUSSION

The binding of chlorogenic acid to fraction 1 protein did not follow the classical Scatchard model where all binding sites are equal and independent. The Scatchard Plot of chlorogenic acid binding to fraction 1 protein was nonlinear and exhibited negative cooperativity (Figure 2). It is possible that the initial binding of chlorogenic acid to fraction 1 induces a conformational change in the protein making additional lower affinity binding sites accessible. A straight line extrapolation of the initial slope to intercept the abscissa gives a value of eight, which is an estimate of the total number of initial binding sites on fraction 1 for chlorogenic acid. Fraction 1 is composed of eight large and eight small subunits (Gray et al., 1980). Thus, our data suggest that there is one high-affinity binding site for chlorogenic acid on either the small or large subunits of fraction 1. The binding constant calculated from the initial slope of the Scatchard plot was 3.8×10^3 L/mol. This is a greater binding constant than has been reported for most hydrophobic protein–ligand interactions (Damodaran and Kinsella, 1980; Arai et al., 1970) and suggests that another (other) mechanism(s) may be involved in chlorogenic acid binding to fraction 1.

Temperature affected the binding of chlorogenic acid to fraction 1 protein. More chlorogenic acid was bound per mole of protein as the temperature was increased from 10 to 40 °C (Figure 3). At 10 °C there appeared to be a maximum of 4 moles of ligand bound/mol of protein. This may indicate that at 10 °C the structure of fraction 1 is stabilized and fewer binding sites are accessible to the ligand, chlorogenic acid. One possible explanation for an increase in the binding of chlorogenic acid to fraction 1 protein as the temperature increases is that a temperature-induced conformational change in the oligomeric structure of fraction 1 occurred. Conformational changes in polypeptides are known to alter their binding affinity for small ligand molecules, e.g., cooperative binding of oxygen to hemoglobin (Koshland et al., 1966) and ligand-induced changes in BSA (Damodaran and Kinsella, 1982).
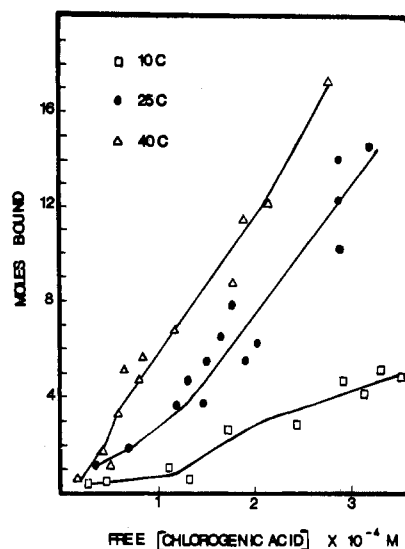

Figure 3. Effect of temperature on the binding of chlorogenic acid to fraction 1 protein. The temperatures used were 10 (□), 25 (●), and 40 °C (△). All solutions contained 0.02 M, pH 7.0, sodium phosphate buffer with 0.02% sodium azide.
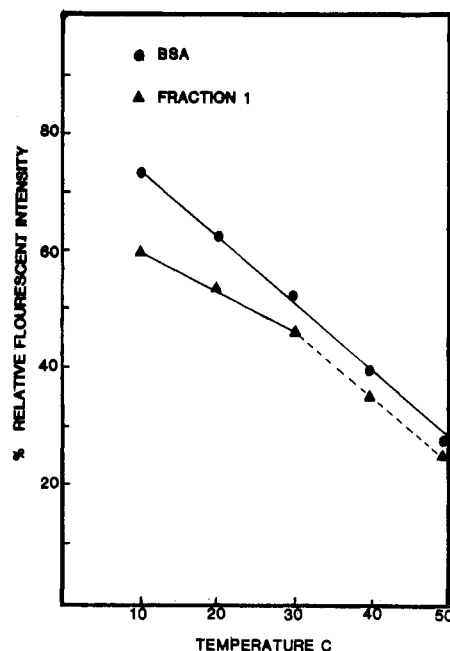
Figure 4. Plot of the relationship between temperature of fluorescence emission intensity of bovine serum albumin BSA (●) and fraction 1 protein (▲). Fluorescent spectra of both proteins were taken at 10-deg temperature intervals from 10 to 50 °C. Protein concentrations were 0.1% in 0.02 M, pH 7.0, phosphate buffer.

Such conformational changes may involve either or both the small and large subunits of fraction 1 protein.

Fluorescent emission spectra are sensitive indicators of changes in the conformation of macromolecules. The quantum yield in fluorescence is itself temperature dependent. As temperature increases fluorescent emission intensity decreases (Guilbault, 1967). In the case of proteins, an abrupt departure from a linear decrease in fluorescent quantum yield with increasing temperature would suggest that a conformational change has taken place. Therefore, we examined the fluorescent spectra of BSA and fraction 1 protein at 10-deg temperature intervals from 10 to 50 °C (Figure 4). When the fluorescent emission intensity of BSA is plotted as a function of temperature, a straight line relationship is obtained. However,
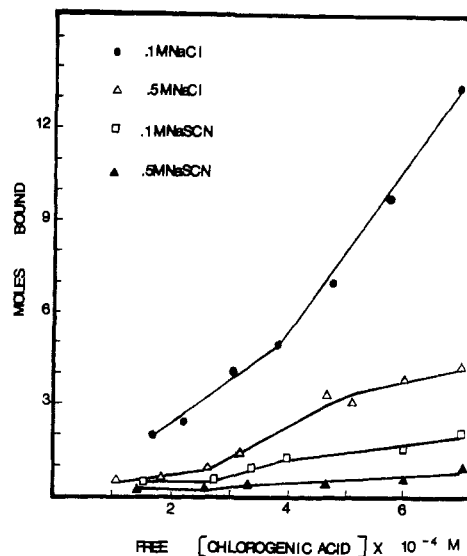
FREE [CHLOROGENIC ACID] X 10⁻⁴   M
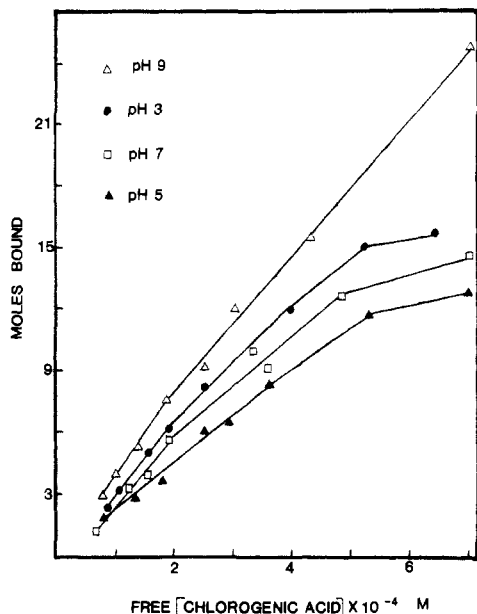
Figure 5. Effect of pH on the binding of chlorogenic acid to fraction 1 protein at 25 °C. All solutions contained 0.15 M NaCl with 0.02% sodium azide and were adjusted to the desired pH with 0.2 N HCl or 0.2 N NaOH.

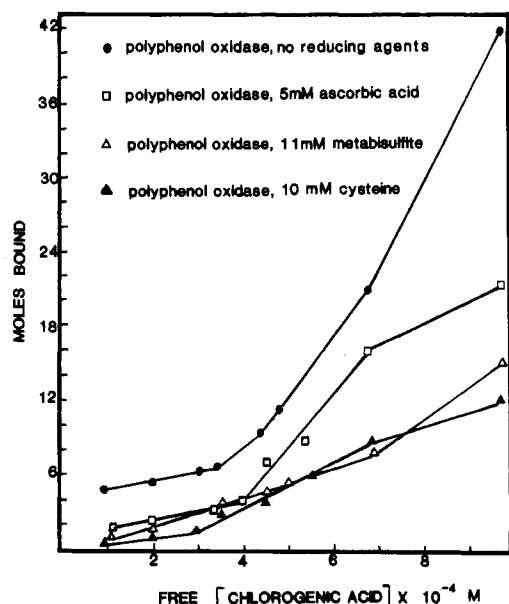FREE   [CHLOROGENIC ACID] x   10⁻⁴ M

Figure 6. Effect of ionic strength on the binding of chlorogenic acid to fraction 1 protein at 25 °C. All solutions contained 0.02 M sodium phosphate, pH 7.0, and 0.1 or 0.5 M of NaCl and NaSCN. (●) 0.1 M NaCl; (▲) 0.5 M NaCl; (□) 0.1 M NaSCN; (▲) 0.5 M NaSCN.

the fluorescent spectra of fraction 1 protein at the same temperature showed a greater than expected decrease in fluorescent emission intensity above 30 °C, indicating that a conformational change(s) in fraction 1 protein may have occurred. This conformational change is undoubtably not due to dissociation of the protein subunits or major unfolding of the individual polypeptide chains because a much larger decrease in fluorescent quantum yield would be expected in such cases (Steiner and Edelhoch, 1961).

There is some evidence in the literature to support a temperature-induced conformational change in fraction 1 protein. Kawashima and Ayabe (1972) reported that the solubility of fraction 1 from tobacco in salt-free solutions decreased from 1.0 mg/mL at 0 °C to 0.2 mg/mL at 40 °C. Badger and Andrews (1974) monitored temperature-dependent changes in the enzymatic activity of fraction 1 protein from spinach and observed a greater than 2-fold increase in the relative oxygenase/carboxylase activity of fraction 1 as the temperature was raised from 10 to 30 °C.

Binding isotherms of chlorogenic acid to fraction 1 protein at four different pH values as shown in Figure 5. The binding of chlorogenic acid appears to be increased at pH 9. The pK_as of the phenolic OH groups of chlorogenic acid are near pH 9 (Nakanishi et al., 1964). At pH 9 it is possible that autoxidation of the ionized phenolic groups on the phenyl ring occurs via a semiquinone radical(s) to form o-quinones. Because of delocalization of electrons on the phenyl ring, this o-quinone form of chlorogenic acid is susceptible to nucleophilic attack by free thiol or amino groups on proteins. Covalently cross-linked tannin protein complexes are believe to be formed in this manner (Pierpont, 1969). The binding of chlorogenic acid to fraction 1 protein decreases to a minimum at pH 5, close to the isoelectric point of the protein, and then increases again at pH 3 (Figure 5). Several investigators have observed an increase in the binding of polyphenols to proteins at pHs below 5. Binding at low pH is generally attributed to hydrogen-bond formation between phenolics and proteins (Sabir et al., 1974). However, electrostatic or hydrophobic interactions may also be involved. Below its isoelectric point a protein carries a net positive charge and therefoe would tend to

attract and bind anions such as the carboxylate anion of chlorogenic acid in this pH range. At pHs close to the isoelectric point hydrophobic associations are possible because of minimum charge repulsion.

The effect of ionic strength of two salts, NaCl and NaSCN, on chlorogenic acid binding to fraction 1 protein is shown in Figure 6. Increasing ionic strength with NaCl dramatically decreased chlorogenic acid binding to fraction 1 protein. Chlorogenic acid has a carboxylic acid group that is predominately ionized at neutral pH. If electrostatic interactions are important in chlorogenic acid binding, then increasing the ionic strength would tend to neutralize charge interactions between chlorogenic acid and positively charged side chain groups (e.g., $E-NH_2$-lysine) on the protein. Wishnick and Lane (1970) reported that increasing ionic strength markedly reduced the binding of the substrate ribulose 1,5-bisphosphate to fraction 1 protein. They presented evidence that at high ionic strength (0.25 M) only four of a maximum of eight binding sites on fraction 1 protein are then available for substrate binding.

Chlorogenic acid is an amphiphile with a hydrophobic phenyl ring at one end of the molecule and a carboxylic acid group and a OH-substituted cyclohexane ring of a more hydrophilic nature at the other end. Thus, it is conceivable that hydrophobic interactions between chlorogenic acid and fraction 1 protein can take place. Experimentally, one would expect a decrease in hydrophobic interactions following the addition of a chaotropic salt like NaSCN that disrupts hydrophobic interactions (Damodaran and Kinsella, 1982). The data presented in Figure 6 do indeed show a decrease in chlorogenic acid binding with NaSCN when compared to the same concentrations of NaCl. This suggests that hydrophobic interactions are important in the binding of chlorogenic acid to fraction 1 protein. However, NaSCN itself is believed to have a greater binding affinity for proteins than salts such as NaCl (Von Hippel et al., 1973). It is possible that NaSCN also competes with chlorogenic acid for similar binding sites on fraction 1 protein or by binding to the protein NaSCN changes the charge distribution on the protein, making chlorogenic acid binding more difficult.

The enzyme polyphenol oxidase catalyzes the oxidation of phenols to quinones. The quinones thus formed may

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Figure 7. Effect of polyphenol oxidase and inhibitors or reducing agents on the binding of chlorogenic acid to fraction 1 protein at 40 °C. All solutions contained 0.02 M sodium phosphate buffer, pH 6.5, with 0.02% sodium azide.

undergo self-condensation and polymerization reactions to form melanin-like polymers (Hathaway and Seakins, 1957). Quinones can also react covalently and noncovalently with proteins. Chlorogenic acid is one of the natural substrates for polyphenol oxidase activity in plant products (Pierpont, 1969; Shalom et al., 1977). The effect of polyphenol oxidase on chlorogenic acid binding in the absence and presence of PPO inhibitors or reducing agents on binding is summarized in Figure 7. Addition of the enzyme prior to equilibrium dialysis caused a more than 2-fold increase in chlorogenic acid binding at the highest concentration of free ligand. When potassium metabisulfite or L-cysteine was included with the enzyme, chlorogenic acid binding was reduced by one-third or more. At lower free ligand concentrations L-ascorbic acid decreased chlorogenic acid binding to levels comparable to the other two reducing agents; but at higher ligand concentrations it was not as effective. Ascorbic acid functions in this system not as an enzyme inhibitor but in maintaining polyphenols, e.g., chlorogenic acid, in the reduced state. In so doing ascorbic acid is slowly oxidized to dehydroascorbic acid and in this form is unable to act as a reducing agent. Higher concentrations of ascorbic acid may be more effective at preventing polyphenol oxidase activity and reducing interactions of phenols with proteins.

If hydrophobic interactions and H bonds are the driving forces for chlorogenic acid binding to fraction 1, then binding should be reduced in the presence of urea (Nozaki and Tanford, 1963). Thus, urea was used as a dissociating agent to disrupt noncovalent interactions between chlorogenic acid and fraction 1 protein. The results (Figure 8) provide an estimate of noncovalent association and covalent bonds. These results suggest that covalent interaction between chlorogenic acid and fraction 1 occurred primarily under two experimental conditions, i.e., when active polyphenol oxidase was present or at pH 9. The pK_a of the most easily ionizable OH group of chlorogenic acid is about pH 8.6 (Nakanishi et al., 1964). At pH 7.0 this phenolic OH group (pK_a 8.6) was calculated as being approximately 3% ionized. Thus, it is possible that even at pH 7.0 some autoxidation of the ionized species of chlorogenic acid occurred. The quinone thus formed could subsequently interact covalently with thiol or amino groups
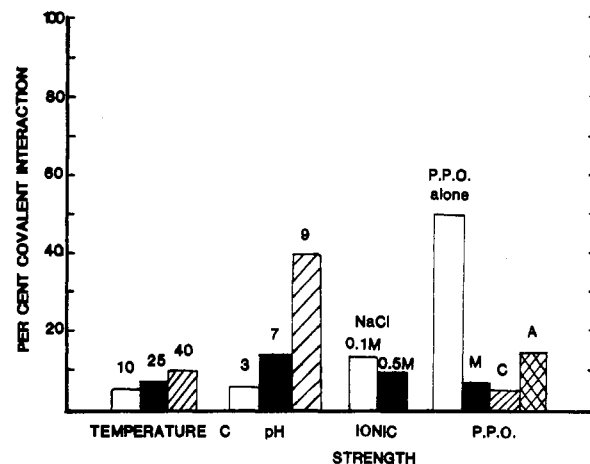
Figure 8. Estimation of the percent covalent interaction between chlorogenic acid and fraction 1 protein under various experimental methods. PPO = polyphenol oxidase present; A = polyphenol oxidase with 5 mM ascorbic acid; M = polyphenol oxidase with 11 mM metabisulfite; C = polyphenol oxidase with 10 mM cysteine.

on fraction 1 protein during the equilibrium dialysis studies performed. This would no longer represent a true ligand–protein interaction.

The present study revealed that the binding of chlorogenic acid to fraction 1 protein is complex and involves noncovalent as well as covalent interactions. The data presented here suggest that electrostatic interactions are important but hydrogen bonds and hydrophobic interactions may also lay a role in the binding of chlorogenic acid to fraction 1 protein. Temperature-induced conformational changes in fraction 1 may significantly increase chlorogenic acid binding. We have also estimated that significant covalent polyphenol–protein interactions may occur under certain conditions. Such interactions may also take place in leaf tissue juice during leaf protein isolation.

Some present methods employed for fractionation of leaf proteins coagulate the chloroplastic fraction II proteins by heating at approximately 60 °C (Edwards et al., 1975; Knuckles et al., 1980). Our results suggest that heating leaf tissue juice may be detrimental from the viewpoint of increasing undesirable polyphenol–leaf protein interactions. One of the goals of our present research on fraction 1 protein is to devise a feasible and economical method for isolation of a food-grade protein(s) from green leaves. Present results suggest that manipulation of parameters such as temperature, pH, and ionic strength are important factors in minimizing polyphenol binding.

This study employed a model system using a single polyphenol, chlorogenic acid. Chlorogenic acid is not truly representative of the entire class of polyphenolic compounds present in green leaves. Further studies are needed to elucidate the mechanisms by which other phenolic compounds may interact with leaf proteins and affect their functional properties and nutritional value.

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Formation of Aroma Components from Nonvolatile Precursors in Passion Fruit

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Thermal treatment at native pH value during simultaneous distillation–extraction of passion fruit pulp significantly increased the concentration of a series of monoterpene hydrocarbons, alcohols, and oxides. GLC–MS investigation of a $CHCl_3$ extract led to the identification of 3,7-dimethylocta-1,5-diene-3,7-diol, 3,7-dimethylocta-1,7-diene-3,6-diol, 3,7-dimethyloct-1-ene-3,7-diol, and 3,7-dimethyloctene-3,6,7-triol. In model experiments the role of these nonvolatile constituents as precursors of a spectrum of volatile terpenoids in passion fruit could be demonstrated. Isolation of a glycosidic fraction on a C_{18} reversed-phase adsorbent and following enzymatic and acid hydrolysis revealed that linalool, nerol, geraniol, and α-terpineol are not present in passion fruits in the free form but rather are present in the bound, glycosidic form. Thermal acid-catalyzed treatment liberates these monoterpene alcohols and leads to transformation into a complex spectrum of passion fruit aroma components.

The flavor composition of yellow (*Passiflora edulis f. flavicarpa*) and purple (*Passiflora edulis* Sims) passion fruits has been investigated intensively in the past few years. Murray et al. (1972), Parliment (1972), and Winter and Klöti (1972) gave a first insight into the complex mixture of aroma components of this tropical fruit. Degradation products of carotenoids (Whitfield et al., 1973, 1977; Näf et al., 1977; Demole et al., 1979; Winter et al., 1979b), sulfur-containing components (Winter et al., 1976), and unusual aliphatic esters (Winter et al., 1979a) were reported to play important roles in the unique and delicate flavor.

The techniques of aroma isolation applied in these investigations ranged from vacuum steam distillation and extraction with organic solvent (Parliment, 1972) to collection from headspace and adsorption on Tenax GC (Chen et al., 1982).

In the present study we wanted to demonstrate the effects of isolation procedures on the flavor composition of passion fruits. To elucidate these influences we did not limit our investigations to the spectrum of volatiles but extended them to the field of nonvolatile components. This combination revealed the relationship between deg-

radation of nonvolatile (glycosidic) precursors and the liberation of volatile aroma components.

EXPERIMENTAL SECTION

Materials. Yellow passion fruits (*P. edulis f. flavicarpa*) were obtained in full ripe state by air freight from Brazil and were stored at 4 °C until analyzed.

Isolation of Volatiles. A total of 600 g of passion fruit pulp was homogenized with 1000 mL of distilled water for 30 s. The homogenate was cleared by filtration through a muslin cloth in a Hafico tincture press at 400 atm. This clarified juice, possessing a pH value of 3.0, was divided into two equal portions. The pH of 0.5 was adjusted to 7.0 by adding an aqueous solution of sodium hydroxide (1 N). The volatiles of both portions were isolated by means of simultaneous distillation–extraction at atmospheric pressure in a modified Likens-Nickerson apparatus using pentane–ether (1:1) for 2 h (Schultz et al., 1977). The aroma extracts were dried over Na_2SO_4, concentrated to a volume of 0.3 mL by using a Vigreux column, and investigated by capillary GLC–MS.

Isolation of Nonvolatile Components. A total of 1.5 kg of passion fruit pulp was homogenized with 500 g of NaCl. The slurry was filtrated through a muslin cloth in a Hafico tincture press at 400 atm and the clarified juice was liquid–liquid extracted with $CHCl_3$ for 24 h. After evaporation of the solvent the residue was taken up in H_2O (5 mL) and washed with pentane (3 × 10 mL). The

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