Chlorogenic Acid–Protein Interactions in Sunflower

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Phenolic compounds in Commander, Majak, and Valley sunflowers varied between 3.0 and 3.5 g of chlorogenic acid per 100 g of flour. Under neutral and alkaline conditions, sunflower protein solutions develop dark green and brown colors because of bonding with oxidation products of polyphenolic compounds, especially chlorogenic acid. Therefore, a reducing agent was utilized in the present study to inhibit the formation of covalent bonds but 30% of the chlorogenic acid was nondialyzable and remained bound to the flour constituents. About one-half of the phenolic constituents were extracted with the soluble sunflower proteins by neutral salt solutions and about one-third of this fraction was also nondialyzable. Fractionation of the neutral salt extracts revealed that all of the soluble chlorogenic acid was associated with the low molecular weight components (mol wt ≤5000) in the third fraction on Sephadex G-25 and fraction V in Sephadex G-200 chromatography. The elution behavior on Sephadex gels, the low nitrogen contents, and the low amino acid recoveries demonstrated that these fractions were polypeptides and oligonucleotides. Rechromatography of fraction V on Sephadex G-200 in the presence of a strong hydrogen bonding agent, 7 *M* urea, revealed that 68% of the salt-soluble polypeptides were hydrogen bonded and about 32% were apparently covalent bonded to chlorogenic acid in Commander sunflower.

Sunflower is one of the fastest developing oilseed crops and ranks second in importance as a world source of vegetable oil. The fibrous by-product of oil extraction is utilized primarily as a protein supplement in livestock rations. However, the flour obtained from the fat extraction of dehulled seed has potential as protein concentrate for human nutrition. In addition to the hulls, sunflower meals contain high levels of phenolic compounds and simple reducing sugars which may contribute dark colors to processed and cooked foods. Investigations are underway to remove these deleterious compounds from the flour by extraction and plant breeding.

Chlorogenic acid is the principal color-forming compound in sunflower kernels but small quantities of caffeic acid and other polyphenolic compounds are also present (Milic *et al.*, 1968; Mikolajczak *et al.*, 1970). The solubilities of chlorogenic acid in methanol, ethanol, and water were reported to be 15.2, 6.2, and 0.6 g/100 ml of solution at 20°, respectively (Sondheimer, 1964). These polar organic and aqueous solvents have been used to remove the phenolic compounds from sunflower kernels, meals, and isolates. Most investigators have demonstrated that the complete removal of phenolic compounds from sunflower meal or isolate required long periods of refluxing or shaking with 50–95% ethanol or 70–85% methanol (Smith and Johnsen, 1948; Joubert, 1955; Mikolajczak *et al.*, 1970; Gheyasuddin *et al.*, 1970; Pomenta and Burns, 1971). Aqueous diffusion removed over 90% of the chlorogenic acid from the dehulled kernels and the defatted flour retained its white appearance under alkaline conditions (Sosulski *et al.*, 1972). With continuous diffusion at 80°, the extraction time was reduced to less than 1 hr but the volumes of aqueous solvent were too high for commercial application of the process (Sosulski *et al.*, 1973).

Studies were then undertaken to identify the factors which contributed to the poor solubility and slow extractability of the phenolic constituents in the sunflower kernel. Sabir *et al.* (1973) determined that chlorogenic acid was bound to the low molecular weight (mol wt) proteins which constituted only 15% of the salt-extractable proteins in the sunflower flour. The hydrogen bonding between the hydroxyl groups of phenolic compounds and the peptide bonds in proteins is known to be unusually strong and the equilibrium in aqueous solution strongly favored the formation of complexes (Loomis and Battaile, 1966). Therefore, the complete extraction of chlorogenic acid from sunflower flour with polar organic, aqueous, or aqueous-organic solvents would be difficult to achieve.

The objectives of the present study were: (i) to determine the quantity of bound and unbound chlorogenic acid among protein fractions; (ii) to characterize the types of linkage between the low molecular weight proteins and chlorogenic acid.

EXPERIMENTAL SECTION

For quantitation, the phenolic acids in the defatted flours of Commander, Majak, and Valley sunflowers were extracted by refluxing the samples with absolute methanol for 5 hr (Smith and Johnsen, 1948).

The proteins and phenolic compounds were extracted from each sunflower variety with 2.5% neutral salt solution (2.5% sodium chloride, 0.02 *M* sodium phosphate buffer (pH 7.0), and 0.01 *M* 2-mercaptoethanol) and fractionated by gel chromatography, as previously described (Sabir *et al.*, 1973). The phenolic constituents in the neutral salt extracts were separated from the high molecular weight proteins and other compounds on a low porosity Sephadex G-25 gel. The flow rate was maintained at 60 ml/hr. The interactions due to hydrogen bonding between proteins and phenolic compounds were investigated by the use of 7 *M* urea, a strong hydrogen bond breaking agent. Fraction V, which was separated on a Sephadex G-200 column, was resuspended into 7 *M* urea in the neutral salt solution and rechromatographed on a short Sephadex G-200 column equilibrated with the same 7 *M* urea solution. The column fractions were scanned between 220 and 350 nm to detect the absorbance maxima of proteins and phenolic compounds.

The dilute column fractions were dialyzed to remove interfering compounds and the protein content was estimated by the spectrophotometric method of Groves *et al.* (1968). The nitrogen contents of salt extracts from flours and the dialyzed and freeze-dried column fractions were estimated by the micro-Kjeldahl procedure (AOAC, 1970). The amino acid analyses of these samples were carried out on a Beckman Model 120C analyzer according to the two-column procedure of Spackman *et al.* (1958). Hydrolyses of the protein samples were conducted with 6 *N* HCl at 110° for 24 hr under vacuum in sealed tubes.

The phenolic acids in the methanol and neutral salt ex-

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Table I. Concentrations of Total and Bound Phenolic Compounds in Sunflower Flours, Salt Extracts, and Third Fraction before (+) and after (−) Dialysis (g of Chlorogenic Acid/100 g of Flour)

Samples	Commander		Majak		Valley	
	Total (+)	Bound (−)	Total (+)	Bound (−)	Total (+)	Bound (−)
			Concentration of Phenolic Compounds			
Flour	3.2	1.0	3.5		3.0	
Salt extract	1.8	0.6	1.9	0.5	1.7	0.4
Third fraction	1.8	0.3	1.7	0.2	1.7	0.2
			Color at pH 10			
Flour		Green				
Salt extract		Pale green		Greenish		Yellow
Third fraction		White		White		White

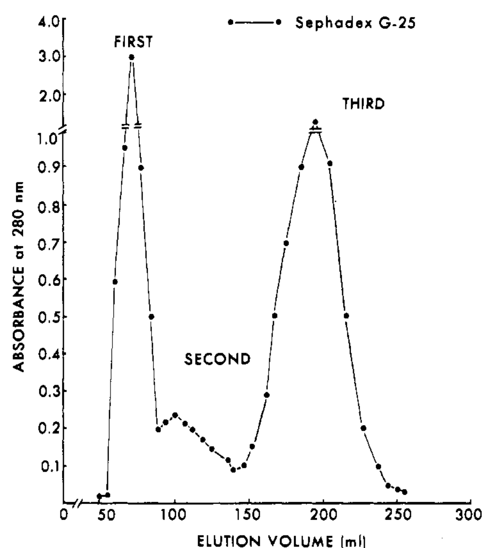

Figure 1. Sephadex G-25 fractionation of salt-extractable Commander proteins on a 2.5 × 40 cm column with a neutral salt solution.

tracts of the flours, dialyzed flours, and extracts, and the column protein fractions were determined as chlorogenic acid by the spectrophotometric procedure of Moores *et al.* (1948). The standard curve was prepared with pure chlorogenic acid in methanol at the absorption maximum of 328 nm.

A seamless cellulose dialysis tubing of average flat width 1.3 in. (Fisher Scientific Co.) was used to separate the bound and unbound phenolic constituents in sunflower flour, neutral salt extracts, and column protein fractions. The dialysis was conducted for 72 hr at 4° against distilled water containing 0.01 *M* 2-mercaptoethanol.

RESULTS AND DISCUSSION

Numerous investigators have demonstrated the presence of chlorogenic acid as the major phenolic acid in sunflower meals (Gheyasuddin *et al.*, 1970; Milic *et al.*, 1968; Sosulski *et al.*, 1972). In the present study the total phenolic compounds, measured as chlorogenic acid, varied between 3.0 and 3.5 g/100 g of flour for the three varieties, Commander, Majak, and Valley (Table I).

To estimate the proportions of free and bound chlorogenic acid the flour and subsequently the salt extracts and their Sephadex protein fractions were dialyzed against distilled water containing a reducing agent, 0.01 *M* 2-mercaptoethanol. When polyphenols are maintained in a reduced state, they may participate in hydrogen bonding with the amide oxygen in the peptide bond, but the probability of their oxidation into quinones and formation of covalent bonds with the proteins is reduced to a minimum (Loomis and Battaile, 1966). In the presence of this reducing agent, dialysis removed 70% of the chlorogenic acid as well as other phenolic compounds (mol wt <12,000) from the Commander flour (Table I). Although chlorogenic acid has a mol wt of only 354.6, a substantial portion remained bound in the dialyzed flour which gave a green color when the suspension was adjusted to pH 10.

About 70% of the sunflower proteins were extracted from these flours with neutral salt solutions (Sabir *et al.*, 1973) but only 55% of the phenolic constituents were soluble in this solvent (Table I). Two-thirds or more of the chlorogenic acid in the neutral salt extract was dialyzable, the remainder being bound to high molecular weight compounds. After dialysis in the presence of the reducing agent the salt extracts showed only yellow to pale green colors at pH 10, which would be predicted from the low chlorogenic acid levels of 0.4–0.6 g/100 g of the original flour.

The nondialyzed salt-soluble proteins were then fractionated on a Sephadex G-25 gel to isolate the chlorogenic acid bound complex (Figure 1). The eluents were collected in 5-ml volumes and the components absorbing at 280 nm were found to be separated in three peaks. The first fraction appeared to be the proteins which were too large for entry into the small pore gel. A second fraction was poorly resolved from the first fraction and occurred partially in the void volume. These would be low molecular weight proteins and other compounds which absorb at 280 nm. The first two fractions showed absorption peaks at 280 nm and no absorption maxima at 328 nm. The large third fraction had absorption peaks at 255 and 328 nm which corresponded to the absorption maxima of oligonucleotides and pure chlorogenic acid, respectively. All of the chlorogenic acid in the neutral salt extracts was eluted with the third fraction (Table I) which was partially within the fractionation limit of the Sephadex G-25 gel (mol wt 1000–5000). Therefore, essentially all of the extractable polyphenolic compounds were associated with only low molecular weight nitrogenous compounds in the neutral salt extract and not with the proteins.

The salt-extractable nitrogen constituted 5.4 g of the original 8.8 g/100 g of flour from the Commander sunflower (Table II). The first fraction was relatively rich in protein, being about 46%, but also contained high molecular weight carbohydrates that did not diffuse through the pores in the G-25 gel. At least 90% of the nitrogenous compounds were proteins and their amino acid compositions were similar to that of the original flour. The proteins in the first fraction contained high levels of methionine and glutamic acid.

The second fraction contained a lower proportion of nitrogen compounds relative to the first fraction but, again, these were primarily proteins (Table II). This fraction was low in lysine and nearly devoid of methionine.

The nitrogen content of the third fraction was very low and the total recovery of amino acids accounted for only 80% of the nitrogen present in this fraction (Table II). Therefore, polypeptide chains rather than proteins were

Table II. Nitrogen (N) and Amino Acid Composition of Salt-Extractable Proteins from Commander Flours and Protein Fractions from Sephadex Gel Chromatography (g of Amino Acid/16 g of N)

Amino acid	In flour	In G-25 fractions			In G-200 fraction V[a]
		First	Second	Third	
% N in flour or protein fraction	5.4	7.4	4.3	2.3	1.4
Essential for human nutrition					
Isoleucine	3.9	4.0	4.3	3.7	3.9
Leucine	6.0	5.8	6.4	5.7	5.2
Lysine	3.3	3.1	2.1	1.9	1.8
Methionine	1.9	3.4	Trace	1.8	2.0
Phenylalanine	4.3	4.6	4.3	4.4	4.7
Threonine	2.9	2.5	2.9	2.6	2.2
Valine	4.8	4.5	4.8	3.9	4.7
Nonessential for human nutrition					
Alanine	4.0	3.3	4.0	3.4	3.5
Arginine	8.9	8.6	9.6	8.0	6.7
Aspartic acid	8.8	8.0	10.5	9.3	9.2
Glutamic acid	23.5	26.1	25.3	20.5	24.2
Glycine	5.6	5.2	4.6	4.1	4.0
Histidine	2.1	1.9	2.4	2.0	2.1
Proline	4.1	4.3	3.8	3.2	4.0
Serine	3.4	3.3	4.1	3.8	3.5
Tyrosine	2.6	2.7	2.0	1.2	1.9
Total	90.1	91.3	91.1	79.5	83.6

[a] From Sabir *et al.* (1973).

present in the third fraction and their amino acid compositions were similar to the low molecular weight (<5000) fraction V obtained by Sabir *et al.* (1973) from the same variety. Although not presented in the table, the oligonucleotide content of the third fraction was found to be 20% of the sample nitrogen. The presence of chlorogenic acid as well as carbohydrates, bound minerals, etc., would account for the low level of total nitrogen in this fraction.

The polypeptides in the third fraction were eluted between 150 and 250 ml in the Sephadex G-25 fractionation (Figure 1) which was partially below the total volume of the bed. Therefore, their molecular weights appeared to be 1000 or less. If the polypeptides were bound to chlorogenic acid, the complex might be retarded on the bed and thus show a lower apparent molecular weight than the free polypeptides. Adsorption between the aromatic ring of the phenolic compound and the Sephadex dextran gels is well documented in the literature (Brook and Munday, 1970).

However, dialysis of the third fraction indicated that only one-sixth of the total chlorogenic acid was bound to other constituents in the salt extract after fractionation. Although dialysis and G-25 chromatography are equivalent processes in many ways, a greater proportion of chlorogenic acid was dialyzable from the third fraction than from the original neutral salt extract (Table I). The low nitrogen concentrations in the third fraction might be responsible for weakening the interaction between the chlorogenic acid and the polypeptides or other nitrogenous constituents.

The salt-soluble proteins from Commander sunflower flour were then chromatographed on a 2.5 × 83 cm column of Sephadex G-200 with neutral salt solution to yield five fractions as previously described (Sabir *et al.*, 1973). All the chlorogenic acid was isolated in the low molecular weight fraction V which was eluted below the exclusion limit for this gel (mol wt ⩽5000). The low nitrogen level and the poor amino acid recoveries (83%) demonstrated the polypeptide nature of fraction V (Table II). This fraction V was used for further investigation on the occurrence and nature of the chlorogenic acid-polypeptide bond.

A strong hydrogen bonding agent such as 7 *M* urea will effectively dissociate other compounds which are bound to proteins or polypeptides with this type of bond (Loomis and Battaile, 1966; Wolf, 1972). However, urea will not compete with phenolic compounds which are complexed by covalent bonds. Therefore, polypeptides and other constituents in fraction V were refractionated on a short Sephadex G-200 column and eluted with a neutral salt solution containing 7 *M* urea. Compounds absorbing at 280 nm were measured in the eluents and these appeared as two peaks which were designated as subfractions V_1 and V_2 (Figure 2). Subfraction V_1 was pooled and scanned at 220 and 350 nm and showed the characteristic absorption maximum of 280 nm for proteins or polypeptides (Figure 3). Subfraction V_1 accounted for 68% of the polypeptides in fraction V as determined by the spectrophotometric procedure of Groves *et al.* (1968). It appeared that this subfraction was associated with chlorogenic acid *via* the hydrogen bond. After dissociation of the complex by 7 *M* urea, the free polypeptides would not adsorb on the Sephadex and a higher molecular weight was indicated by the lower elution volume than was obtained for fraction V. In addition, the urea treatment may have denatured the polypeptides and the altered molecular configuration could account for the apparent increase in molecular weight of subfraction V_1.

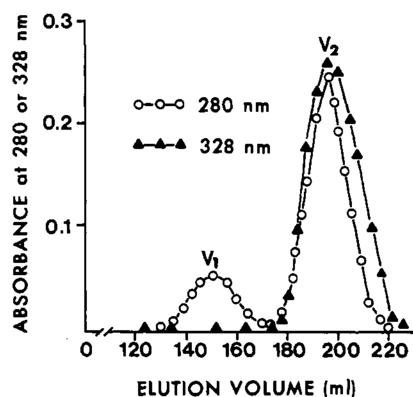

Figure 2. Sephadex G-200 rechromatography of salt-extractable Commander polypeptide fraction V on a 2.5 × 37 cm column with 7 *M* urea in the neutral salt solution.

Subfraction V_2, which constituted 32% of the fraction V polypeptides according to the Groves *et al.* (1968) proce-

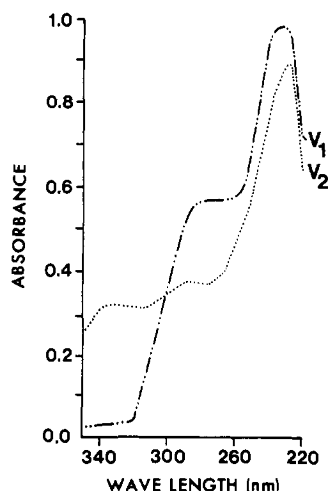

Figure 3. Ultraviolet absorption spectra of salt-extractable Commander polypeptide subfractions V_1 and V_2.

dure, appeared below the fractionation limit of the gel (Figure 2). Subfraction V_2 showed absorption at 328 nm as well as 280 nm and the ultraviolet scan showed absorption peaks at both wavelengths (Figure 3). Since 7 *M* urea did not dissociate the bonds between the chlorogenic acid and the polypeptides in subfraction V_2, covalent bonds appeared to be present. Reifer and Augustyniak (1968) have also reported the presence of covalent bonds between low molecular weight nitrogen compounds and chlorogenic acid in sunflower.

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Absorption, Translocation, and Metabolism of Metribuzin (BAY-94337) in Sugarcane

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Radioactivity from root-absorbed metribuzin, labeled with ^{14}C at position 5 in the ring, was deposited mainly in the leaves as unknown metabolites. Less than 10% of the ^{14}C could be accounted for and measured by gas chromatography as the parent herbicide and three known metabolites; acid hydrolysis increased recovery to about 10%. At 12 weeks, 84% of the radioactivity initially added to the nutrient solution was recovered—75% in the plant tissue, including abscised leaves, and 9% in the nutrient medium. Foliar treatment resulted in a distal movement within the leaves of 5-20% of the applied ^{14}C; another 10-20% remained at the treated site and 1-2% appeared proximally to the treatment area. Foliar residues averaged 25% of the amount applied, relatively independent of the length of time after surface treatment.

Metribuzin (BAY-94337), 4-amino-6-*tert*-butyl-3-(methylthio)-1,2,4-triazin-5(4*H*)-one, is a representative of a new class of *as*-triazinone herbicides (Eue *et al.*, 1969) with selective utility for sugarcane. We describe its absorption when applied to the foliage and, in nutrient culture solution, to the roots of sugarcane. The herbicide was diluted with radioactive metribuzin in order to follow translocation of the parent compound and its metabolites. Patterns of distribution and recovery of radioactive residues over a 12-week period are compared with those of the *s*-triazine herbicides atrazine and ametryne (Hilton *et al.*, 1970); with 2,4-D (Ashton, 1958); and with picloram (Hilton *et al.*, 1973). We determined by gas chromatography the known metabolites (DA, DK, and DADK) in various parts of the sugarcane plant and related their amounts to the radioactivity present in the same samples.

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MATERIALS AND METHODS

Root Application. Sugarcane cuttings of cultivar H50-7209 were established as small, uniform plants in individual containers in 3 l. of aerated Hoagland nutrient solution. To each container was added 29,630 μg of unlabeled metribuzin and 370 μg of metribuzin-^{14}C (labeled in position 5 of the ring, specific activity 1.4 mCi/mmol), resulting in herbicide concentrations of 10 μg/ml in contact with the plant roots at the start of the experiments. All plants were exposed to outdoor conditions; water and nutrient were added as needed to maintain volume.

Plants were removed after 1, 4, 8, and 12 weeks of continuous treatment and separated into green leaves, stalk, the apical meristem of the primary shoot, vegetative seedpiece, secondary basal shoots (suckers) if present, the ac-