The Effect of Chlorogenic, Quinic, and Caffeic Acids on the Solubility and Color of Protein Isolates, Especially from Sunflower Seed[1]

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ABSTRACT

Chlorogenic (CGA), quinic (QA), and caffeic (CA) acids have been identified as components of sunflower seed. CGA in particular has been indicted as causing the discoloration of sunflower meal at alkaline pH and the broad pH range (3 to 7) of minimum solubility and maximum precipitability of sunflower protein. The objective of this research was to test the validity of these indictments. Two approaches were used in evaluating the solubility effect. First, pH-solubility and precipitation profiles of sunflower meal freed from CGA, QA, and CA were compared with the original meal. Second, samples of a commercial soybean-protein isolate were treated with the organic acids individually, and their solubility profiles compared with that of untreated soybean-protein isolate. The results indicate that the unusual solubility profile is a characteristic of the sunflower protein, and not induced by interaction with any of the three organic acids. To measure the chromophoric effects of the three acids, soybean-protein isolate treated with each was extracted with water at pH values ranging from 2.0 to 11.0, and the colors noted visually. For comparison, sunflower meal was treated in a similar manner. The CGA-treated soybean meal exhibited color changes similar to the sunflower meal, although the colors obtained were lighter. QA had no effect on the color; and CA produced a slight pink, rather than the green to brown of CGA. The lyophilized CGA-treated soybean meal had a grayish color, whereas those treated with QA and CA were similar to the control. This suggests that chlorogenic acid *per se* darkens the protein.

In 1897, Osborne and Campbell (1) recognized the presence of an organic compound in sunflower seed which they named helianthotannic acid. They attributed the dark color of their protein preparation from sunflower seed to this phenolic acid. Gorter (2) identified the compound as chlorogenic acid, and in 1947 Rudkin and Nelson (3) determined its structure as an ester of quinic and caffeic acids. It is now recognized that chlorogenic is widely distributed in higher plants and in some cases is present in substantial concentration (4).

On hydrolysis, chlorogenic (CGA) acid yields quinic (QA) and caffeic (CA) acids. Both QA and CA have been shown to be present in sunflower seed (5,6).

The involvement of chlorogenic acid in the production of a dark color in the protein isolated from sunflower seed by precipitation of a 10% NaCl extract was first proposed by Osborne and Campbell (1). Later, Smith and Johnsen (7) reported that the production of green- and brown-colored proteins by extraction of sunflower-seed meal at pH 9.5 and 11.5, followed by isoelectric precipitation, was due to CGA. These latter authors also advanced the hypothesis that the low water solubility and the broad pH range (3 to 7) of minimum solubility and maximum precipitability of sunflower protein are probably due to the formation of a protein-CGA complex.

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The objective of the present investigation was to examine the validity of these indictments and to determine whether QA or CA (or both) might also be involved.

MATERIALS AND METHODS

The sunflower seed used in this investigation was of the Armavirec variety grown at Hillsboro, Texas, in 1968. Sunflower meal was prepared from the dehulled seeds by cold hexane extraction and by air desolventization. CGA, CA, and QA were purchased from the Sigma Chemical Company. A commercial soybean protein isolate (Promine-D) was received as a gift from the Central Soya Company.

Analysis for CGA, CA, and QA

The spectra of the two polyphenolic acids were determined with a Beckman DU spectrophotometer equipped with a Gilford attachment. Standard curves for CGA in 5% methanol and for CA in 95% ethanol were prepared at various concentrations at 324 and 320 nm., respectively. The standard curve for QA was prepared according to the method described by Mesnard and Devaux (8).

R_f values of the three organic acids were determined by ascending paper chromatography on Whatman No. 1 chromatographic paper (18 X 12 in.) in *n*-butanol-acetic acid-water 4:1:5 (v./v./v.) (BAW) as the developing solvent system. The chromatograms were developed at 25°C. for 18 hr. The spots of CGA and CA were located by an ultraviolet fluorescent lamp and that for QA after the paper was sprayed with periodate-2 thiobarbituric acid.

For the extraction of the organic acids, duplicate 2.5-g. samples of the sunflower meal were boiled 10 min. with an isopropanol-water azeotrope (88:12, w./w.) to inactivate the enzymes. The meal-azeotrope suspension was quantitatively transferred to a 25 X 80-mm. cellulose extraction thimble supported in the neck of a 500-ml. Erlenmeyer flask by a thin glass rod. The filtrate was collected. The residue was washed successively with the following solvents according to Wilson et al. (9): isopropanol-water (1:1, v./v.); isopropanol-benzene-methanol-water (2:1:1:1, v./v./v./v.); and isopropanol-water azeotrope (88:12, w./w.).

The combined filtrate, washings, and extracts were evaporated to dryness *in vacuo* at 36°C. (±1°) on a rotary evaporator.

For comparative purposes, duplicate 5.0-g. samples of the meal were extracted with 95% ethanol, as described by Milić et al. (5).

Duplicate 0.5-ml. aliquots from each extract were applied as streaks on previously washed Whatman No. 1 chromatographic paper and dried by blowing with cold air. The chromatograms were developed with BAW and, after 18 hr., were dried in air.

Preliminary identification was made by comparing R_f values and confirmed by spectrum determination and co-chromatography. The bands were cut out of the chromatograms and then eluted with an isopropanol-water azeotrope in a Soxhlet extractor for 12 hr. The quantities of the organic acids were determined by comparison against the standard curves.

Solubility Profiles

Two approaches were taken to determine the effect of the organic acids upon the solubility profile of the proteins in sunflower-seed meal. First, the nitrogen-solubility profile and precipitation profile of sunflower-seed meal were

compared with those obtained for a meal from which the organic acids had been extracted. Second, samples of a commercial soybean-protein isolate were treated with these organic acids individually and their nitrogen-solubility profiles compared with that of untreated soybean-protein isolate.

The organic acids were removed from the sunflower meal by a slight modification of the method described by Joubert (10). Defatted meal (200 g.) ground to pass a 60-mesh screen was extracted repeatedly with 50:50 alcohol-water mixture until the extract gave only a faint yellow color with NaOH solution. The meal was then extracted repeatedly with cold distilled water in a Waring Blendor, followed by centrifugation at 2,000 × g. Finally, the meal was extracted with acetone and washed with anhydrous ethyl ether. All of the operations were carried out in a cold room at about 6°C. The meal was air-dried.

The meal was tested for CGA by a NaOH solution (0.2%), with which CGA normally produces a chrome-yellow color; and by the Hoepfner (11) method, which depends on the production of an intense carmine red color when CGA or CA in acetic acid solution reacts with an alkaline nitrite. A faint yellow color with the former and no color with the latter test indicated that for all practical purposes the meal was free from CGA and CA. A test of the meal by the method of Mesnard and Devaux (8) indicated that the meal was free of QA.

The treatment of the soybean isolate was as follows: 0.708 g. of CGA, 0.36 g. of CA, and 0.38 g. of QA were each dissolved in 200-ml. portions of water, producing 0.01M solutions of the acids. These solutions were added to individual samples of 25 g. each of the soybean isolate. The mixtures were made into homogeneous slurries by stirring for one-half hour. A control sample was prepared by making a slurry with water. All samples were then freeze-dried.

Nitrogen content of the samples was determined by the Kjeldahl method. For the determination of nitrogen-solubility profiles, 1.0-g. samples were extracted with 50.0 ml. of pH-adjusted water. The pH was adjusted and maintained at the desired level by the addition of 0.5N HCl and 0.5N NaOH solutions during each extraction. The increase in volume as a result of pH adjustment was noted and accounted for in all subsequent calculations. Clear extracts were obtained by centrifugation at 4,600 × g for one-half hour, followed by filtration when necessary.

In determining the pH-nitrogen precipitation profile, 25.0 g. of the sample was extracted at pH 11.0 for 1 hr. Clear extracts were obtained by two successive centrifugations, one at 2,000 × g followed by a second at 4,600 × g. Aliquots (25.0 ml.) of the extracts were pipetted into 50-ml. centrifuge tubes. The pH of the extract was adjusted with 0.5N HCl and the resulting precipitate was separated by centrifugation at 4,600 × g and the clear supernatant liquid was poured directly into Kjeldahl flasks. Nitrogen contents of the various extracts and supernatant liquids were determined by the Kjeldahl method and the results are reported as the percentage of total nitrogen extracted.

Determination of the Chromophoric Behavior of CGA, QA, and CA in the Presence of Protein

Soy-protein isolate was treated separately with 0.01M solutions of CGA, CA, and QA as described above. Samples (1.0 g.) of the various acid-treated isolates were extracted separately with 50 ml. of water at pH values ranging from 2.0 to 11.0. The colors were noted visually. For the sake of comparison, the color changes exhibited by the sunflower meal under the same conditions of treatment were also noted.

RESULTS AND DISCUSSION

The levels of CGA, CA, and QA extracted by the procedure of Wilson et al. (9) from this sample of Armivirec sunflower-seed meal were as follows: CGA, 2.7%; QA, 0.38%; and CA, 0.2%.

The CGA content is substantially higher than that reported by Milić et al. (5) for the Sunrise variety. To determine whether this difference was due to variety or to the difference in the efficiency of the extraction procedures employed, duplicate analyses were made with the extraction procedures described by Milić et al. (5). By the latter procedure, both the CGA (1.2%) and the QA (0.12%) were substantially lower.

It appears that the ethanol extraction procedure is not sufficiently efficient to extract all of the CGA and QA. Additional evidence that this is the case is furnished by the positive Hoepfner test and the production of a chrome-yellow color by the addition of 0.2% NaOH. On the other hand, these tests were negative with the residual meal following extraction by the procedure of Wilson et al. (9).

The inability of the milder procedures to extract all of the organic acids may be explained by assuming that a large part of the acids are rather strongly bound to some particulate system and as a consequence are not extracted unless an exhaustive extraction procedure is employed. This observation has important practical significance in any attempt to devise a procedure to produce a sunflower-protein product devoid of its customary chromophoric properties.

It is of interest to note that the CGA content as determined by the ethanol-extraction procedure is close to that value reported by Milić et al. (5) for

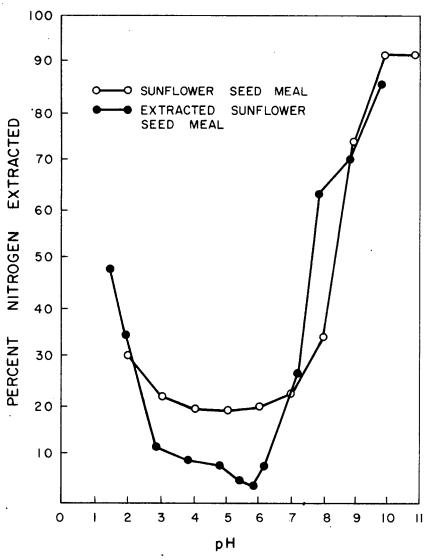


Fig. 1 (left). Nitrogen-solubility profile of sunflower-seed meal before and after removal of CGA, CA, and QA by extraction.

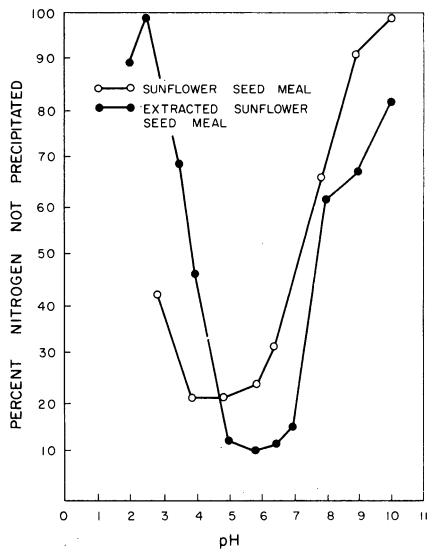


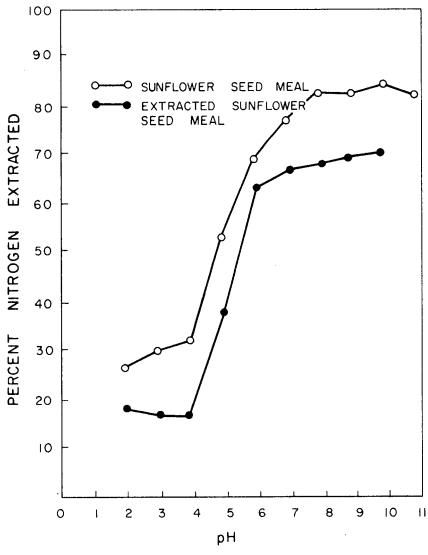
Fig. 2 (right). Nitrogen-precipitation profile of sunflower-seed meal before and after removal of CGA, CA, and QA by extraction.

the meal from the Sunrise variety of sunflower seeds, although the QA content is lower. As also observed by Milić et al. (5), the CA band was almost invisible on the chromatogram of the ethanol extract.

The effect of the extraction of the three organic acids from sunflower-seed meal by the Joubert (10) method on the solubility of the protein (nitrogen) is shown in Figs. 1 through 3. This extraction procedure is rather rigorous and it would be anticipated that some alteration in the properties of the protein might result. The major effect noted in Fig. 1 is the reduced amount of nitrogen products extracted in the pH range 3 to 7. It is evident that the nitrogen compounds soluble in water in this range are largely extracted by the procedure used to remove the organic acids. However, this did not alter the characteristic broad range of minimum solubility of the sunflower meal.

On precipitation from solution, the sunflower-seed proteins do not exhibit quite as broad a range of minimum solubility. The precipitation curves for the extracted meal again reflect the loss of soluble nitrogen compounds in the range of pH 5 and up. There is an unexpected apparent increase in the solubility of the extracted meal at the lower pH values.

Figure 3 shows the effect of the removal of CGA, CA, and QA from sunflower-seed meal on its solubility in 1.0M NaCl. Again the major effect appears to be the loss of the more soluble nitrogen compounds. The lowered solubility in



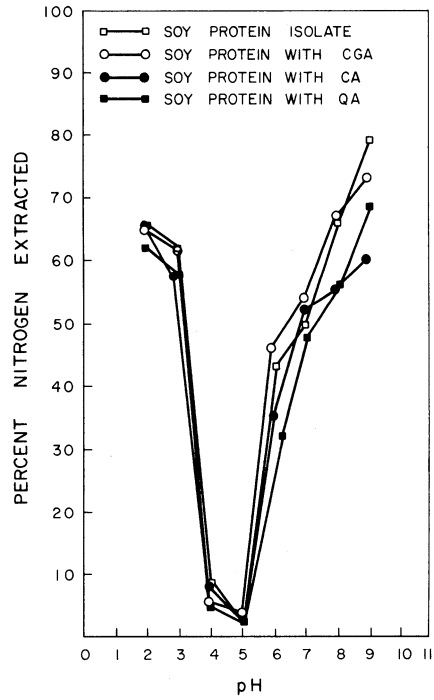


Fig. 3 (left). Nitrogen-solubility profile of sunflower-seed meal in 1.0M NaCl before and after removal of CGA, CA, and QA by extraction.

Fig. 4 (right). Nitrogen-solubility profiles of soybean-protein isolate before and after treatment with CGA, CA, and QA.

TABLE I. EFFECT OF pH ON THE COLOR OF THE EXTRACTS FROM SUNFLOWER-SEED MEAL, SOYBEAN-PROTEIN ISOLATE, AND SOY ISOLATE TREATED WITH CGA, CA, AND QA

			Soybean-Protein Isolate		
			Treated with		
pH	Sunflower-Seed Meal	Untreated	CGA	CA	QA
2,3,4	Colorless	Colorless	Colorless	Colorless	Colorless
5	Slightly dark	Colorless	Colorless	Colorless	Colorless
6	Slightly dark	Colorless	Slightly dark	Colorless	Colorless
7	Slightly yellow	Creamy	Slightly yellow	Slightly yellow	Creamy
8	Chrome yellow	Slightly yellow	Yellow	Yellow with some pink	Slightly yellow
9	Green	Slightly yellow	Greenish	Slightly pink	Slightly yellow
10	Green	Slightly yellow	Green	Slightly pink	Slightly yellow
11	Brown	Slightly yellow	Slightly brown	Slightly pink	Slightly yellow

the alkaline range suggests some degree of protein denaturation resulting from the extraction procedures employed.

Figure 4 shows the nitrogen-solubility profiles of soybean-protein isolate before and after treatment with CGA, CA, and QA. It is evident that treatment with these acids under the conditions employed has had very little effect upon the solubility profiles of soybean protein. Some reduction in solubility in the alkaline ranges is suggested, but could only be confirmed by a rather substantial statistical experiment which is beyond the intent and scope of this research.

The purpose of the present investigation was to determine whether the unusual solubility profile of sunflower-seed proteins was induced by the presence of CGA. The evidence contradicts this allegation and suggests that this is an inherent characteristic of the sunflower proteins.

On the other hand, the data in Table I demonstrate that CGA is clearly responsible for the chromophoric properties of sunflower seed. The color changes shown for sunflower-seed meal at different pH values are in agreement with earlier reports (1,7). A chrome-yellow color was obtained at pH 8 to 9 which turned deep green within a few minutes. Above pH 11, the initial yellow turned into a dark brown without passing through the intermediate green.

The soybean-protein isolate treated with about 2.8% CGA exhibited color changes similar to those of the sunflower meal, although the color obtained at each stage was lighter than that of the sunflower meal. The soybean protein treated with 1.4% CA had some chromophoric properties of a rather mild nature. This very mild effect of CA was somewhat surprising inasmuch as in aqueous solution it changes rather rapidly from yellow to green and from green to brown at pH values above 9.0. On the basis of presently available knowledge, it is difficult to offer a reasonable explanation for the difference in the chromophoric behavior of CA in aqueous solution and CA-treated protein.

The soybean-protein isolate treated with QA did not differ from the control in chromophoric properties under various pH conditions.

These observations support the position that CGA is the agent responsible for the chromophoric behavior of sunflower-seed meal; QA is undoubtedly not involved; and CA is possibly not involved, although its reaction in aqueous solution tends to keep it under suspicion. It is of interest to note that the lyophilized CGA-treated soybean-protein isolate had a slight gray color. Those treated with CA and QA had colors similar to the control. This indicates that CGA darkens protein, even at slightly acidic conditions.

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