Low Molecular Weight Proteins from Sunflower (*Helianthus annuus* L.) Seed: Effect of Acidic Butanol Treatment on the Physicochemical Properties

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Sunflower seed protein interactions with chlorogenic acid (CGA) result in unfavorable color development. CGA is shown to be more associated with the low molecular weight proteins (LMW). This can alter the properties of the protein, especially the LMW proteins. The sedimentation coefficient of the LMW proteins remained unchanged at 1.8 S. The protein resolved into four peaks on Sephadex G-75 gel filtration. The composition of the second fraction increased from 35% to 53% upon acidic butanol treatment. The last fraction had higher amounts of CGA. The native polyacrylamide gel electrophoresis indicated fast moving bands, but the molecular weights of the three major bands did not change, as seen in SDS-polyacrylamide gel electrophoresis as a result of acidic butanol treatment. The secondary structure of the protein was 19% α-helix, 40% β-structure, and 41% aperiodic for the native and 27% α-helix, 38% β-structure, and 35% aperiodic for the acidic butanol treated protein. The fluorescence emission maximum indicated a red shift from 330 nm, indicating structural alterations of the proteins.

INTRODUCTION

Sunflower seeds, besides yielding oil, are also a potential source of vegetable proteins. They are less utilizable for human consumption because of the unfavorable color development resulting from protein interaction with chlorogenic acid (CGA) at neutral or alkaline pH (Pierpoint, 1969a). Such interactions are a result of oxidation of CGA by polyphenol oxidase or other nonenzymatic reactions giving rise to the formation of *o*-quinones, which are highly reactive and spontaneously undergo oxidation and form covalent bonds with the functional groups of proteins such as amines, thiols, thioethers, indole, imidazole, and disulfide groups (Shuttleworth, 1966; Loomis and Battaile, 1966; Loomis, 1969; Pierpoint, 1969a, 1970). Most of the CGA is associated with the low molecular weight proteins, of which 68% is hydrogen bonded and 32% covalently linked (Sabir et al., 1974a); only 10% of the phenolics is associated with high molecular weight protein components (Raymond et al., 1981).

The low molecular weight proteins of sunflower seeds constitute nearly 20% of the total proteins and are termed albumins (Joubert, 1955; Schwenke and Raab, 1973; Schwenke et al., 1973; Rahma and Narasinga Rao, 1979). Several methods for isolation of the low molecular weight protein fraction by complexing with tannins, dextran sulfate, and polyphosphates (Schwenke and Raab, 1973; Schwenke and Simon, 1974; Schwenke et al., 1975) and treatment with solvents and acidic butanol (Joubert, 1955; Sodini and Canella, 1977; Rahma and Narasinga Rao, 1979; Sripad et al., 1982; Prasad, 1988) have been reported. These methods aim at the removal of chlorogenic acid from the protein.

Washing with acidic butanol, besides reducing the chlorogenic acid content, could also induce changes in the physicochemical properties of low molecular weight proteins. These changes could alter the surface properties, fluorescence emission spectrum, secondary structure, and electrophoretic properties of the proteins. The analysis of these proteins gives an indication of the extent of variation in properties of the proteins itself as a result of acidic butanol washing.

To compare the effect of acidic butanol treatment on the low molecular weight proteins, the proteins were isolated from the untreated and the butanol treated flour and compared for their chemical, physicochemical, and conformational changes, and the results are reported in this paper.

MATERIALS AND METHODS

Sunflower seeds of the genus *Helianthus annuus* L. variety A-1 were procured from the local market in Mysore, Karnataka, India. Chemicals used were from the following sources: bovine serum albumin, ovalbumin, lysozyme, acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine (TEMED), 2-mercaptoethanol, guanidine hydrochloride, Tris, chlorogenic acid, sodium dodecyl sulfate, and Coomassie brilliant blue R-250 were from Sigma Chemical Co., St. Louis, MO; Sephadex G-75 was from Pharmacia Fine Chemicals, Uppsala, Sweden; bromophenol blue and sucrose were from Koch Light Laboratories, Colnbrook, Bucks, England; ammonium persulfate, amido black 10B, Methyl Cellosolve, and ninhydrin were from E. Merck, Darmstadt, Germany; calcium chloride was from BDH, Bombay, India; sodium hydroxide was from Astra-IDL, Bangalore, India. The dialysis tubings were from Thomas Scientific Co., Swedesboro, NJ. All other chemicals used were of analytical reagent grade.

Estimation of Chlorogenic Acid. (*a*) *Using Water.* The extraction of chlorogenic acid from the defatted flour was done by washing the defatted flour at 1:20 (w/v) with distilled water. The pH of the slurry was adjusted to 5.0. The slurry was stirred for 15 min and filtered through a Büchner funnel. The process was repeated until the chlorogenic acid in the water extract was not detectable with subsequent washings as monitored by its absorbance at 324 nm and also by the procedure described by Pomenta and Burns (1971).

(*b*) *Using Acidic Butanol.* The chlorogenic acid from the flour was extracted with acidic butanol as described by Sodini and Canella (1977).

The untreated low molecular weight proteins are termed LMW-P and the acidic butanol treated proteins LMW-PB.

Isolation of the Low Molecular Weight Fraction. Dehulled sunflower seeds were flaked and defatted with *n*-hexane in a column defatter until the fat content was less than 1%. They were dried at 40 °C in a cabinet dryer for 12 h and powdered in

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a quadramat mill to obtain a flour of 150-mesh size. The flour obtained from either untreated or acidic butanol treated seeds was mixed with phosphate buffer (pH 6.0, 0.02 M) containing 1 M NaCl in the ratio 1:10 (w/v) and stirred using a magnetic stirrer for 1 h. The contents were centrifuged at 6000*g* for 30 min, and one part was dialyzed against extraction buffer and is termed total protein. To the other part was added slowly powdered ammonium sulfate to reach a concentration of 30% (w/v) of ammonium sulfate. It was stirred over a magnetic stirrer for 1 h and centrifuged at 6000*g* for 30 min. The ammonium sulfate concentration in the supernatant was further raised to 40% (w/v). It was stirred for 1 h and centrifuged at 6000*g* for 30 min. The precipitate was dissolved in phosphate buffer (pH 6.0, 0.02 M) containing 1 M NaCl and dialyzed extensively against distilled water, lyophilized using a Virtis Freeze Mobile-6, and stored at −10 °C in a desiccator until required for further experiments.

Determination of Absorption Coefficient. The nitrogen content of the isolated protein was converted to protein by using a conversion factor of 5.7 (AOAC, 1984). From a plot of absorbance at 280 nm (using a Shimadzu UV 150-02 spectrophotometer) vs protein concentration the absorbance of a 10 mg/mL protein solution was calculated and expressed as the absorption coefficient of the sample.

Absorption Spectra. The absorption spectra of the proteins were recorded in a Beckman DU-8B recording spectrophotometer in the range 240–350 nm using a 1 cm path length quartz cell at 27 °C with the lambda-scan software supplied with the instrument.

Amino Acid Analysis. The amino acid composition of the protein was determined in a LKB-Alpha amino acid analyzer equipped with a programmer and integrator. The analysis was done according to the procedure of Moore and Stein (1963).

Gel Chromatography. Gel filtration of the low molecular weight protein fraction was performed on Sephadex G-75 (superfine) gel in phosphate buffer (pH 6.0, 0.02 M) containing 1 M NaCl in a column of 1.8-cm diameter by 100-cm length with a flow rate of 20 mL/h. The samples were dialyzed for 24 h in the same buffer. A total of 5.4 mL containing 190 mg of low molecular weight protein from the untreated flour and a total of 4 mL containing 250 mg of low molecular weight protein from the acidic butanol treated flour were loaded on the column. Fractions of 3 mL/tube were collected, and the absorbance was monitored at both 280 and 324 nm for proteins and chlorogenic acid, respectively, in a Shimadzu UV 150-02 double-beam spectrophotometer.

Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE was performed in 10% acrylamide with 0.4% bis-(acrylamide) as the cross-linking agent in 0.02 M, pH 8.3, Tris-glycine buffer. Protein samples of 10–100 μg were loaded after a prerun of 30 min. The electrophoresis was performed at a constant current of 4.5 mA/tube until the dye reached the bottom of the gel. The proteins were fixed in 20% trichloroacetic acid and stained in 0.1% Coomassie brilliant blue R-250. The gels were destained in 7.5% (v/v) acetic acid.

Sedimentation Velocity. Analytical ultracentrifugation measurements were performed with a Spinco Model E analytical ultracentrifuge equipped with a rotor temperature indicator, control unit, and phase plate schlieren optics. A standard 12-mm Kel F cell centerpiece was used. Protein solutions in the concentration range 1–2% were used for the experiments, and runs were made at 59 780 rpm at 27 °C. Photographs were taken at regular intervals of time. $S_{20,w}$ values were calculated according to the procedure described by Schachman (1959).

Circular Dichroism. The ultraviolet circular dichroic spectra were recorded using a Jasco J 20-C recording spectropolarimeter at 27 °C. The far-ultraviolet spectra were recorded in 205–260-nm wavelength range using a 0.1-cm quartz cell with a protein concentration of 0.2 absorbance at 280 nm, while the near-ultraviolet spectra were recorded in 260–345-nm range using a 1.0-cm quartz cell with a protein concentration of 1.0 absorbance at 280 nm.

Fluorescence Spectra. The fluorescence excitation and emission spectra of the proteins were recorded in an Aminco-Bowman spectrofluorometer at 27 °C. Protein solutions with concentrations of 25–35 μg/mL having absorbances of 0.1–0.15 at 280 nm were used for the fluorescence measurements. The excitation spectra were recorded from 200 to 300 nm with emission at 330 nm, and the emission spectra were measured in the range 300–380 nm with excitation at 280 nm. All measurements of fluorescence were monitored after 10 s of excitation.

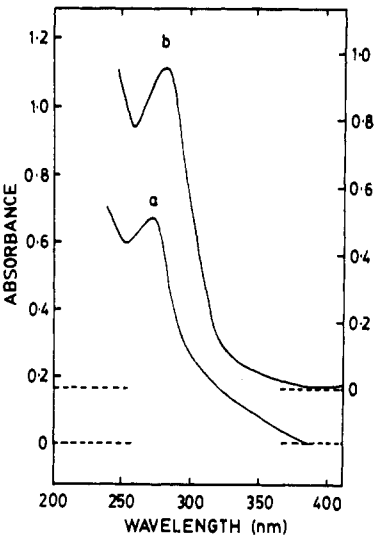

Figure 1. Ultraviolet absorption spectrum from 240 to 400 nm of sunflower seed low molecular weight proteins isolated from (a) untreated and (b) acidic butanol treated defatted flour in 0.02 M, pH 6.0, phosphate buffer containing 1 M NaCl. To compare the spectra (qualitatively), the ordinates are shifted such that there is no overlapping of spectra.

RESULTS AND DISCUSSION

The chlorogenic acid content of the untreated sunflower flour was 3.8% (includes free and bound). The content of chlorogenic acid in LMW-P was estimated to be 0.42 ± 0.05%, and after eight washings with acidic butanol, in the LMW-PB, it was estimated to be 0.13 ± 0.02%.

It has been reported that in spite of several methods being available for the removal of chlorogenic acid with water (Lanzani et al., 1979) or with solvents such as methanol, ethanol, 2-propanol, and acetone and coprecipitation with acid washing (Pierce, 1970; Cater et al., 1972; Sosulski et al., 1973; Sodini and Canella, 1977; Assogna et al., 1979; Lanzani et al., 1979; Schingoethe and Ahrar, 1979; Sripad et al., 1982), the strong covalent interaction of the polyphenols with the proteins as well as the interaction of the oxidation products of polyphenols through amino, thiol, and active methylene groups (Pierpoint, 1969a,b; Ribereau-Gayon, 1972) constitutes a limit to the complete removal of the ligand.

The ultraviolet absorption spectra of the LMW-P and LMW-PB are shown in Figure 1. The LMW-P and LMW-PB have absorbance maxima at 260 and 275 nm, respectively. Thus, acidic butanol washing shifts the absorption maximum of the proteins to 275 nm. CGA by itself has no absorption band at 260 nm, but in the presence of the protein and as a complex, the absorption band is a result of alteration in the absorbing chromophores of the protein. The trailing portion of the spectrum at 350 nm could be due to the remaining ligand possibly bound covalently to the protein.

The amino acid compositions of the LMW-P and LMW-PB are shown in Table I. There is only marginal difference in the amino acid compositions of the two proteins.

In the analytical ultracentrifuge, sedimentation velocity profile and scanner tracing at 415 nm (Figure 2) indicate that CGA is distributed predominantly between the 11S protein fraction and higher amounts in the LMW protein fraction in the total protein profile on a mole to mole basis.

Table I. Amino Acid Composition (Percent) of LMW-P and LMW-BP

amino acid	LMW-P	LMW-PB
lysine	9.84	10.54
histidine	4.68	4.28
arginine	3.06	2.64
aspartic acid	5.65	7.08
threonine	3.71	6.76
serine	24.20	22.57
glutamic acid	12.10	11.37
proline	2.42	2.97
alanine	16.61	13.51
half-cystine	2.26	1.81
valine	5.00	4.61
methionine	0.48	0.82
isoleucine	3.06	3.46
leucine	4.03	5.11
tyrosine	1.13	1.15
phenylalanine	1.77	1.32

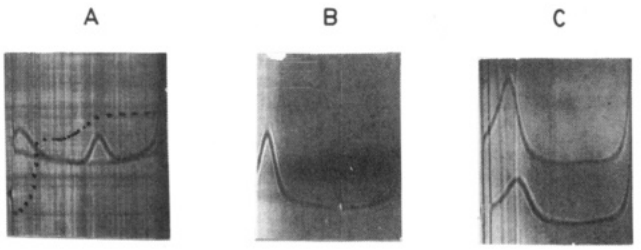

Figure 2. Sedimentation velocity pattern of sunflower seed (A) total proteins, (B) low molecular weight protein fraction from untreated defatted flour, and (C) acidic butanol treated defatted flour at two concentrations in 0.02 M, pH 6.0, phosphate buffer containing 1 M NaCl. The sedimentation run was performed at 60 000 rpm for the total proteins (A) at 20 °C and at 59 780 rpm at 27 °C for the low molecular weight proteins (B and C); the sedimentation proceeds from left to right. The time of photograph was 45 min after two-thirds maximum speed was attained at a bar angle of 60° for (A) and of 70° for (B) and (C). A protein concentration of 10.5 mg/mL was used for (A), while it was 12 mg/mL for (B). Two protein concentrations of 16 (upper) and 8 mg/mL (lower), respectively, were used for the acidic butanol treated low molecular weight proteins (C). The dotted line in photograph A is the scanner tracing of added CGA at 1×10^{-3} M concentration to the protein and scanned at 415 nm during protein distribution of the velocity run.

The sedimentation velocity profiles of the low molecular weight protein fractions from both the untreated and acidic butanol treated flours indicate a broad peak in the LMW-P. Unlike LMW-P, at higher protein concentration in LMW-PB there was a small percentage of high molecular weight protein fraction with a sedimentation coefficient of 16S present (Figure 2).

Such formations of aggregates have been reported by a number of workers in oilseeds as a result of solvent treatment (Sodini and Canella, 1977; Navin Kumar, 1982; Prasad, 1988; Sripad et al., 1982). On extrapolation to zero protein concentration, a value of 1.82 ± 0.05 S was obtained for the sedimentation coefficient of the LMW proteins (Figure 3, inset). Further, the $S_{20,w}$ value does not appear to depend on the concentration of the protein in either sample. This result is in agreement with values reported in the literature, where different procedures were used for isolation of low molecular weight proteins. They are generally termed "2S" protein because of sedimentation coefficients in the neighborhood of 2.0 (Prakash and Narasinga Rao, 1986).

The gel filtration patterns of LMW-P and LMW-PB are shown in Figure 3. The results indicate that the LMW-P are made up of at least four protein fractions (a–d). The last fraction (e) is a polyphenol having higher absorbance at 280 nm since it did not stain for protein in

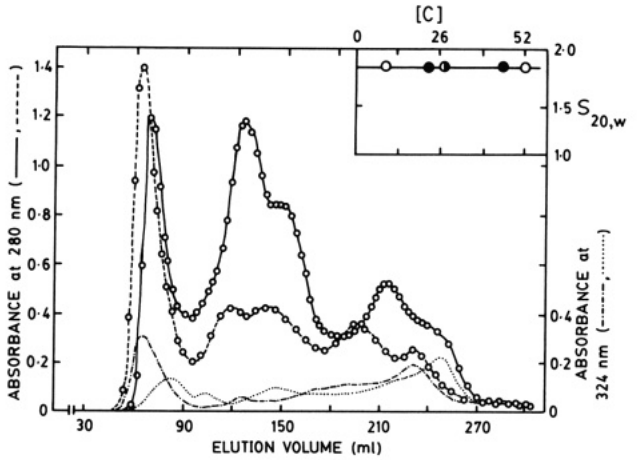

Figure 3. Gel filtration pattern of sunflower seed low molecular weight proteins isolated from the untreated defatted flour and acidic butanol treated flour. The absorbance was monitored at 280 nm for proteins and indicated as - -O- - for the untreated and as – O – for the acidic butanol treated defatted flour extracted in 0.02 M, pH 6.0, phosphate buffer containing 1 M NaCl in Sephadex G-75 gel. The absorbance was monitored at 324 nm for CGA and is indicated as – ● – for the low molecular weight protein from the untreated flour and as ··· for the acidic butanol treated flour. (Inset) Sedimentation coefficient of the low molecular weight proteins as a function of protein concentration. Open circles indicate LMW-P, and shaded circles indicate LMW-PB. The overlap of the data points of the two samples is shown as a half-shaded circle.

the polyacrylamide gel electrophoresis. The percentages of the various fractions are 37, 35, 18, 7, and 3 (Figure 3). The distribution of CGA is more predominant with the fraction eluting at 240 mL (Figure 3), which could be due to the complexing of CGA with the proteins (McManus et al., 1981; Van Sumere et al., 1975). The protein elution profile and the percentages of the various fractions of LMW-PB differ from that of LMW-P. Fractions of 35% in LMW-P increased to 53% in LMW-PB. There was also a corresponding decrease in the percentage of the fraction from 37% to 22%, respectively, in the two proteins. This may be due to the effect of butanol on the possible association–dissociation of the second fraction. Also, the decrease in the fraction eluting at 240 mL could be due to preferential leaching out of this particular fraction to a certain extent during washing treatment or the acidic butanol could have weakened the forces of interaction between the ligand and the protein, as it is known that CGA can interact noncovalently through hydrogen bonding to the proteins. It is known that alcohols, in general, affect the conformation of the proteins (Herskovits and Jaillet, 1969). This can be further enhanced due to aqueous organic solvent systems (Fukushima, 1969). Further, the removal of CGA itself could destabilize the proteins, resulting in changes in the gel filtration pattern, and the solubility of proteins can change due to solvent treatments (Mann and Briggs, 1950; Smith et al., 1951; Rayner and Dollear, 1968; Herskovits and Jaillet, 1969; Rayner et al., 1970; Prasad, 1988).

The SDS–PAGE pattern of the proteins indicated three major bands along with some slow moving minor bands in the two proteins. The molecular weights of the three major bands were 32 500 ± 1000, 24 500 ± 1000, and 19 000 ± 750 and 32 500 ± 1000, 24 500 ± 1000, and 19 500 ± 750 for the upper, middle, and lower bands of LMW-P (Figure 4A) and LMW-PB (Figure 4C), respectively. Thus, treatment with acidic butanol did not cause any significant change in the electrophoretic patterns of the two proteins. Sripad (1984) also did not observe any difference between

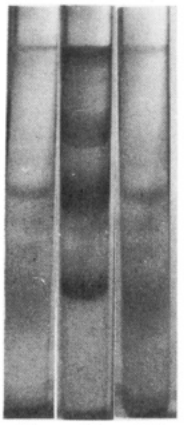

Figure 4. SDS–polyacrylamide gel electrophoretic pattern of the low molecular weight proteins isolated from (A) untreated, (B) standard marker proteins [bovine serum albumin (top band), ovalbumin (middle band), and lysozyme (bottom band)], and (C) acidic butanol treated defatted flour in 0.02 M, pH 8.3, Tris–glycine buffer in 10% acrylamide with 0.4% bis(acrylamide) as the cross-linking agent. The protein concentrations were (A) 50, (B) 60 (with 20 μg of each standard protein), and (C) 70 μg.

solvent treated and untreated samples in SDS–PAGE. The fact that no major change can be seen either in the molecular weight or in the number of polypeptide chains implies that acidic butanol probably removes preferentially CGA present in the flour without affecting significantly the individual polypeptide chains, as can be seen with the pattern of the totally denatured SDS–PAGE (Figure 4). The present data are similar to those reported by Prasad (1988).

The near-ultraviolet circular dichroic spectra (Figure 5A) of the LMW-P indicated a minimum at 268 nm for both proteins. However, for the LMW-PB the rotation increased in the range 268–270 nm. Such changes could be due to changes in the environment of the tryptophanyl residues. In LMW-P there was also present a broad positive peak in the range 310–330 nm which was absent in LMW-PB. Contributions at this wavelength could be due to the ligand, chlorogenic acid. On treatment with acidic butanol this positive band disappeared.

The far-ultraviolet circular dichroic spectrum (Figure 5B) is characterized by a minima at 208 nm and a sharp shoulder at 220 nm for the LMW-P sample having a fair amount of rotation at both wavelengths. Such results, with higher helix content, have been obtained on low molecular weight proteins from other oilseeds (Prakash and Narasinga Rao, 1986; Prakash, 1989; Schwenke et al., 1989). The percentages of secondary structures, as calculated according to the method of Chang et al. (1978), were 19% α-helix, 40% β-structure, and 41% aperiodic structure with an error of ±3%. The peak at 208–210 nm indicates a high content of ordered structure.

In the LMW-PB (Figure 5B), butanol treatment caused an increase in rotation at 220 nm with a more defined shoulder at this wavelength as well as an increase in negative rotations as compared to LMW-P. It has 27% α-helix, 38% β-structure, and 35% aperiodic structure with an error of ±3%. Similar results are reported for a number of seed proteins in the presence of alcohol-like solvents (Prakash and Narasinga Rao, 1986), which, due to their low dielectric constants, can interact with proteins affecting the circular dichroic spectrum (Strickland, 1976) and cause structural changes involving the relative positions of the asymmetric groups and their spatial configuration, thus producing a pronounced change in the rotation (Kauzmann, 1959).

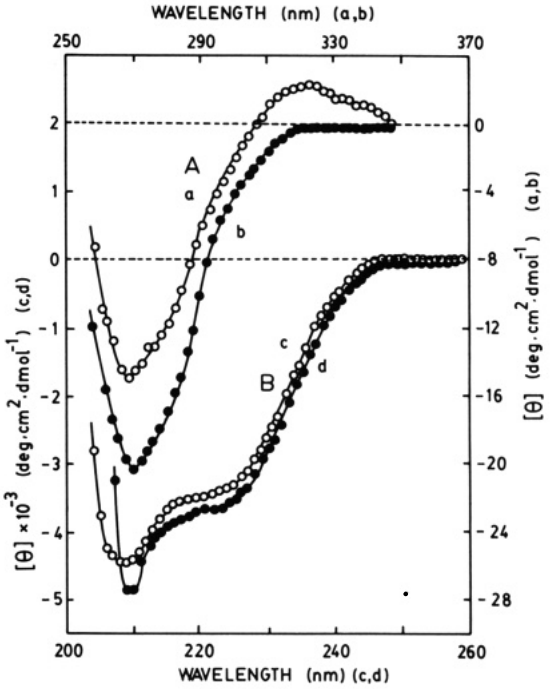

Figure 5. Near- and far-ultraviolet circular dichroism spectra of the low molecular weight protein isolated from untreated and acidic butanol treated defatted flour in 0.02 M, pH 6.0, phosphate buffer containing 1 M NaCl. (A) Near-ultraviolet dichroic measurements were recorded with protein solutions of 0.53 and 0.31 mg/mL for the (a) untreated and (b) acidic butanol treated proteins, respectively, in 1.0-cm cells. The spectra were scanned from 260 to 345 nm. (B) Far-ultraviolet circular dichroic spectra were recorded with protein solutions of 0.05 and 0.03 mg/mL for the (c) untreated and (d) acidic butanol treated proteins, respectively, in 0.1-cm cells. The spectra were scanned from 205 to 260 nm.

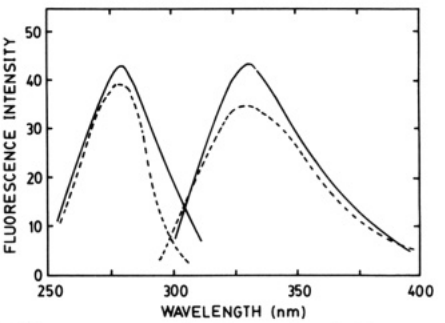

Figure 6. Fluorescence excitation and emission spectra of the low molecular weight proteins isolated from the untreated defatted flour (—) and acidic butanol treated flour (- - -) in 0.02 M, pH 6.0, phosphate buffer containing 1 M NaCl. The excitation wavelength was 280 nm for the emission spectrum, and protein concentrations of 0.05 mg/mL for the untreated protein and 0.03 mg/mL for the acidic butanol treated protein were used. The fluorescence emission spectrum was measured 10 s after excitation of the sample.

The LMW-P and LMW-PB both showed a fluorescence excitation maximum at 280 nm and emission maxima at 330 and 333 nm, respectively (Figure 6). The fluorescence emission maximum of the protein at 330 nm indicates that it is dominated by the presence of tryptophanyl residues in a more nonpolar environment in the protein molecule (Teale, 1960). The 3-nm red shift for LMW-PB could indicate the exposure of some of the tryptophanyl residues to the exterior of the protein due to butanol treatment. However, the change is minimal since tryptophan or its amide has its emission maximum around 345 nm in aqueous phase (Shifrin et al., 1971).

Treatment with acidic butanol has brought about certain subtle changes in the physicochemical properties of the LMW-P as can be seen from the data. However, the protein elution profile on gel filtration indicates major changes. Thus, the effect of butanol on such a system clearly demonstrates two effects: (i) structural changes as monitored by circular dichroism and fluorescence and (ii) association–dissociation phenomena of the proteins as observed by gel filtration, gel electrophoresis, and analytical ultracentrifuge.

It is well understood that the interaction of polyphenols with protein is largely a surface phenomena and takes place in two distinct phases. In the first instance the polyphenol seeks preferred sites and regions on the protein where numerous aromatic rings readily accommodate it by hydrophobic interactions. This may result in preferential solvent exclusion from the protein molecule as a second phase. This stage can firmly be reinforced by proper deployment of hydrogen bonds between phenolic residues and polar groups present in the protein (guanidino, amide, peptide, amino, hydroxyl, and carboxyl groups). This is often termed "docking" of the polyphenol to the polypeptide surface (Spencer et al., 1988). Such a process may result in the formation of a monolayer of polyphenols around the protein molecule, which is a much lesser hydrophilic layer, and may lead ultimately to aggregation and precipitation. This is possible because of the behavior of polyphenols acting as polydentate ligand through several potential sites on the proteins.

Washing with solvents like acidic butanol may disrupt only the first phase of the interaction which is nonpolar (in other words, it may remove polyphenols which are not docked). In this process, the physicochemical properties of the protein could be affected.

Such changes are easily explained by both acidic pH and butanol–protein interaction in terms of altering the physicochemical properties as well as altering the forces between the various protein fractions. The study also demonstrates the preferential interaction of CGA to specific low molecular weight proteins as observed in gel filtration results and scanner run of the analytical ultracentrifuge.

Even though most of the results are explainable in terms of acidic pH and butanol, the role played by CGA in maintaining the integral structure of the protein through hydrogen bonding as well as charge-transfer complex formations cannot be overruled, since the removal of CGA can cause destabilization and changes in protein structure. Even though several workers have isolated different fractions of sunflower seed proteins, it is important, for the understanding of the CGA–protein complexes, that all of the low molecular weight proteins present be isolated to homogeneity and the interaction studied in detail from the point of stoichiometry, nature of binding sites, and thermodynamics of such interactions. However, since CGA is an intrinsic ligand present in the seed, it may pose a difficulty in its complete removal from the protein since it is well-known that part of CGA is covalently bound to these proteins (Sabir et al., 1974b).

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