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Isolation and Characterization of Undenatured Chlorogenic Acid Free Sunflower (*Helianthus annuus*) Proteins

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A method for obtaining sunflower protein (SFP) isolate, nondenatured and free of chlorogenic acid (CGA), has been developed. During the isolating procedure, the extent of CGA removal and protein denaturation was monitored. The defatted flour contained 2.5% CGA as the main phenolic compound. Phenolic compounds were removed by aqueous methanol (80%) extraction, before protein extraction at alkaline pH and diafiltration. Differential scanning calorimetry and solubility tests indicated that no denaturation of the proteins had occurred. The resulting protein products were biochemically characterized, and the presence of protein−CGA complexes was investigated. SFPs of the studied variety were found to be composed of two main protein fractions: 2S albumins and 11S globulins. In contrast to what has been previously reported, CGA was found to elute as free CGA, not covalently associated to any protein fraction.

KEYWORDS: ***Helianthus annuus*; sunflower; protein; denaturation; chlorogenic acid; differential scanning calorimetry; complexes; isolation**

INTRODUCTION

Sunflower seeds are used in the food industry as a source of oil. One of the byproducts of the oil extraction process is sunflower meal, which has a high protein content (40−50%), making sunflower meal an attractive protein source. Furthermore, sunflower protein (SFP) is reported to contain no antinutritional components, such as protease inhibitors, and the amino acid composition of its proteins complies largely with the FAO (Food and Agriculture Organization) pattern with the exception of lysine (*1*). Moreover, SFP consists mainly of albumins and globulins (70−85%) and, therefore, has a high intrinsic solubility. As solubility is a prerequisite for many functional properties, SFP may prove to have high potential for use as a food ingredient.

However, nowadays, the main outlet of SFP is in animal feed. One of the reasons is that during oil production, due to mechanical pressing and solvent extraction at elevated temperatures, protein denaturation occurs, resulting in an insoluble and nonfunctional protein fraction (*2*). Another reason that hampers the application of SFP as a food ingredient is the presence of relatively high amounts of phenolic compounds, especially chlorogenic acid (CGA). Phenolic compounds interact and form complexes with proteins, thereby reducing both their digestibility and their functionality (*3*, *4*). Furthermore, the presence of CGA results in a dark color of SFP products (*5*−*7*). The interaction may become irreversible when, under alkaline conditions, phenolic compounds autocatalytically oxidize to quinones and react with functional protein groups, such as amines, thiols, thioethers, indole, imidazole, and disulfide groups (*8*).

Many methods have been proposed for isolating SFP and removing phenolic compounds from sunflower seeds. They are mainly based on the following principles: (i) extraction with mixtures of organic solvents and water (*5*, *8*−*16*), (ii) extraction with aqueous solutions of acids, salts, or/and reducing agents (*17*−*22*), (iii) membrane filtration (*23*), (iv) precipitation of pigments and nonprotein compounds (*24*−*26*), and (v) combinations thereof (*27*−*32*). Of all of the methods described, the most promising ones with respect to the efficiency of CGA extraction are those that extract phenolic compounds with mixtures of organic solvents and water (*3*, *13*, *33*, *34*). However, a major disadvantage of these methods may be that organic solvent water mixtures are known (*35*−*38*) to cause protein denaturation, which may result in diminished solubility and protein recovery. Methanol, ethanol, and 2-propanol are especially promising with respect to both protein recovery (*34*, *39*) and CGA extractability (*3*, *39*). In contrast, several studies have revealed the protein-denaturing effect (*8*, *14*, *31*, *40*) of butanol and acetone, mainly monitored by the decrease in protein solubility. No information, other than that on solubility properties, is known about the protein-denaturing effect of methanol, ethanol, and 2-propanol mixtures during the CGA removal in sunflower meal.

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To be able to assess the intrinsic properties of SFP as a functional food ingredient, the protein should be both free of CGA and nondenatured. In the research described in this paper, an isolation procedure is set up to meet these requirements. Therefore, during the isolation procedure, the extent of CGA removal, the presence of protein−CGA complexes, and the protein denaturation are monitored. Furthermore, the resulting protein products are biochemically characterized.

MATERIALS AND METHODS

Materials. Dehulled "Mycogen Brand" sunflower seeds were purchased from H.Ch. Schobbers B. V. (Echt, The Netherlands). CGA and caffeic acid (CA) were purchased from Sigma (Zwijndrecht, The Netherlands). Hexane was purchased from Chemproha (Dordrecht, The Netherlands). All other chemicals were obtained from Merck (Darmstadt, Germany).

Preparation of the Defatted Meal (DM). The dehulled seeds were milled in a laboratory grinder (Janke and Kunkel GmbH, Staufen, Germany) for 3 min, avoiding high temperature by cooling the grinder periodically with liquid nitrogen. The resulting meal (named seed meal, SM) was defatted by hexane extraction at room temperature. The meal was extracted 4 times, each during 2 h, using a meal-to-solvent ratio of 1:5 (w/v). The DM was separated by paper filtration (Whatman n° 1) and left to dry overnight at room temperature.

Preparation of the Defatted Dephenolized Meal (DDM). DM was extracted with cold (4 °C) mixtures of organic solvents and water [ethanol 95% (v/v), 2-propanol 70% (v/v), and methanol, 80% (v/v)] at a meal-to-solvent ratio of 1:20 (w/v) by stirring the suspension for 4 h. After the suspension was filtered, the extraction was repeated until the extract no longer developed a yellow color upon addition of NaOH. Finally, the defatted dephenolized protein (DDM) was dried in a vacuum oven at 30 °C overnight.

Chemical Analysis. Moisture and ash content were determined gravimetrically according to the AACC Method 44-15A (*41*) and AACC Method 08-16 (*41*), respectively. The fat content was determined according to the AACC Method 30-25 (*41*). The crude protein content (N × 6.25) of meal and protein products was determined by the Kjeldahl method, AACC 46-12 (*41*). All analyses were carried out at least in duplicate.

Preparation of the Sunflower Isolate (SI). The DDM was suspended in water (1% of protein, w/v) and stirred for 30 min while keeping the pH at 9 by the addition of 1 N NaOH. Soluble protein was recovered by centrifugation (30000*g*, 20 min, 20 °C). The pellet (P) was re-extracted (similar conditions), and the two supernatants were combined to render the extract (E). This extract was subjected to diafiltration using extensive washing. This filtration process was carried out by circulation through a 10 kDa TFF cartridge (Millipore Corp., Bedford, MA). The retentate obtained was subsequently freeze-dried and denoted SI.

Protein Extractability. The protein extractability of DM or DDM was studied as a function of pH. A dispersion of DM or DDM representing 0.5 g of protein in 45 mL of water was stirred for 5 min at room temperature. Then, the pH was adjusted to the desired value by the addition of 1 N NaOH or HCl. Stirring was continued for 1 h, while the pH was monitored every 15 min and readjusted, if necessary. The final volume was adjusted to 50 mL using water. After it was centrifuged (30000*g*, 30 min, 20 °C), the supernatant was filtered to remove floating particles. Aliquots of the supernatant were freeze-dried, and their protein content was determined by Kjeldahl analysis. Extractability measurements were performed at least in duplicate.

Sugar Content. The neutral sugar content and composition of the fractions were analyzed as alditol acetates (*42*). Fractions were subjected to pretreatment with 72% (w/w) H_2SO_4 for 1 h at 30 °C prior to hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C using inositol as an internal standard. Alditol acetates were separated on a DB-225 column [5 m × 0.53 mm internal diameter; film thickness 1.0 μm] (J&W Scientific, Folsom, CA) in a CE Instruments GC 8000 TOP (ThermoQuest Italia, Milan, Italy) and operated at 200 °C and equipped with an FID (ThermoQuest Italia, Milan, Italy) detector set at 270 °C. The uronic acid content was determined according to Thibault (*43*) using glucuronic acid as a standard. In this method, 96% (w/w) H_2SO_4 containing 0.0125 M sodium tetraborate was used in order to quantify glucuronic acid as well as galacturonic acid residues.

Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (*44*) on a Mini-PROTEAN II electrophoresis Cell (BIO-RAD, Veenendaal, The Netherlands), following the instruction of the manufacturer. Protein samples of 10−15 μg were dissolved in either reducing or nonreducing sample buffer and applied to homogeneous 12% gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue. Low molecular weight (LMW) markers ranged from 14 to 94 kDa (Amersham, Pharmacia Biotech, Uppsala, Sweden): α-lactalbumin (14 400), soybean trypsin inhibitor (20 100), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000), and phosphorylase *b* (94 000).

Differential Scanning Calorimetry (DSC). The calorimetric studies were performed using a differential scanning calorimeter Micro-DSC III (Seteram, Caluire, France). A 9% (w/w) protein dispersion in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl was used. Heating was performed at a rate of 1 °C/min over the temperature range of 20−120 °C. The measurements were carried out in duplicate.

Gel Filtration Chromatography. Gel filtration chromatography was performed on an Äkta Explorer System (Amersham, Pharmacia Biotech, Uppsala, Sweden). The protein (5−10 mg/mL) was extracted at room temperature from SM, DM, DDM, and SI with 50 mM sodium phosphate buffer, pH 6.9, containing 0.25 M NaCl. After the extracts were centrifuged, the supernatants were applied directly to a Superose 6 HR 10/30 column and eluted with the same buffer at a flow rate of 0.5 mL/min at room temperature. The eluate was monitored at 214 and 324 nm. Solutions of CGA were analyzed according to the same procedure to determine its elution volume.

Determination of CGA and CA. CGA and CA were extracted by incubating 250 mg of sample with 25 mL of 80% (v/v) aqueous methanol at 60 °C during 1 h. The extraction was performed 4 times. The extracts were filtered, pooled, dried in a GyroVap speed-vacuum (HOWE, Etten-Leur, The Netherlands), and redissolved in 4% acetic acid (v/v) in water. CGA and CA contents were determined by reversed-phase high-performance liquid chromatography (Waters 2690 Separations Module, Etten-Leur, The Netherlands), using a Symmetry C_{18} column at room temperature and at a flow rate of 1 mL/min. The eluent was a mixture of A (4% acetic acid (v/v) in methanol) and B (4% acetic acid (v/v) in water). After isocratic elution during 5 min with 10% A/90% B, the linear gradient to 25% A/75% B in 10 min and to 90% A/10% B in 1 min was used, followed by isocratic elution for 1 min 90% A/10% B. The eluate was monitored at 324 nm. Pure CGA and CA were employed as standards.

RESULTS AND DISCUSSION

Preparation of Sunflower Products. *Defatting.* To remove oil, the dehulled sunflower seeds were extracted with hexane at room temperature. This gentle treatment resulted in a reduction of the fat content from 55 (w/w) to 4% (w/w). As a result, the protein content increased from 26 to 55% (w/w) and the ash content increased from 3.3 to 7% (w/w). The DM contains 2.5% (w/w) CGA and 0.1% (w/w) CA. Similar figures using dehulled seeds have been found previously (*34*, *39*).

Dephenolizing. On the basis of results obtained by other investigators (*5*, *9*, *13*, *28*, *45*−*47*), fixed concentrations of organic solvents in water were tested for their ability to extract phenolic compounds. **Table 1** shows the amounts of CGA and CA extracted with the different solvents used. From this table, it can be concluded that aqueous methanol and 2-propanol are equally efficient with respect to the amount of CGA and CA extracted, whereas aqueous ethanol turned out to be a poor

Table 1. Protein Extractability at pH 7 and pH 10 of the DM before and after Dephenolization by Different Solvents and CGA Extracted by These Solvents

	CGA[a] extractability	CA[a] extractability	protein extractability (%)[b] pH 7	protein extractability (%)[b] pH 10
DM			21 ± 1	79 ± 2
methanol 80%	100 ± 4	100 ± 6	19 ± 2	80 ± 2
2-propanol 70%	88 ± 12	96 ± 8	18 ± 2	72 ± 1
ethanol 95%	24 ± 8	90 ± 4		

[a] Expressed as proportion (%) of extracted CGA or CA. [b] Amount of soluble protein expressed as proportion (%).

Table 2. Enthalpy, Temperature, and Onset Temperature of Denaturation As Measured by DSC for Sunflower Products: SM, DM, DDM, and SI

	onset temp of denaturation (°C)	temp of denaturation (°C)	enthalpy of denaturation (J/g)[a]
SM	95.1 ± 0.2	101.5 ± 0.3	14.5 ± 0.2
DM	95.6 ± 0.3	101. 9 ± 0.1	15.2 ± 0.6
DDM	95.0 ± 0.1	101.1 ± 0.1	14.2 ± 0.5
SI	93.6 ± 0.2	99.7 ± 0.2	14.9 ± 0.4

[a] The enthalpy values have been normalized for the protein content.

extraction solvent for CGA. This finding is in agreement with previous publications (*10*, *48*). Because aqueous methanol and 2-propanol gave the best results, the use of these solvents was examined further.

The treatment of proteins with mixtures of water and organic solvent may lead to protein denaturation and a subsequent decrease in protein solubility. Therefore, the protein extractability of the dephenolized meals in water at two different pH values was determined (**Table 1**). Aqueous 2-propanol clearly reduced protein extractability at pH 10 but only slightly at pH 7, whereas aqueous methanol did not affect the extractability at either value. Therefore, we further examined the effect of aqueous methanol on protein denaturation by DSC. **Table 2** shows the enthalpy, temperature, and onset temperature of denaturation of several sunflower products. In the DSC thermograms, only one endothermic peak appears for the SM, DM, and DDM samples around 100 °C with a similar onset temperature of 95 °C. Moreover, the enthalpy of denaturation per gram of protein does not differ significantly between the samples. This clearly indicates that the protein remained undenatured and is not affected by the treatments with either hexane (for defatting) or 80% (v/v) aqueous methanol (for dephenolizing). Denaturation temperatures found are in agreement with values previously reported (*40*, *49*, *50*). The calorimetric enthalpy of denaturation is similar to the one obtained by Sanchez and Burgos (*40*) but is markedly lower than the value presented by other authors (*49*, *50*). This discrepancy may be due to differences in experimental conditions, such as the buffer used, pH, and protein composition (11S/2S ratio).

The use of 80% (v/v) aqueous methanol does not seem to result in protein denaturation, probably due to the low temperature applied during extraction and the presence of a high methanol concentration in the water mixture. The latter ensures negligible protein solubility, preventing the hydration of proteins and, therefore, the binding of CGA. Subsequently, 80% aqueous methanol was used for CGA removal in the remainder of this study.

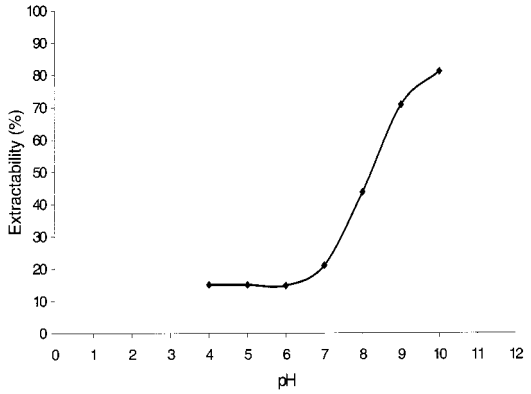

Figure 1. Protein extractability of the DM in water (1% protein, w/v).

Table 3. Yield of the Isolation Procedure and Protein Content of Sunflower Products: SM, DM, DDM, E, and SI

		yield	
	protein content (%)[a]	solids (%)[b]	proteins (%)[c]
SM	26 ± 1	100	100
DM	55 ± 1	46 ± 2	98 ± 2
DDM	66 ± 1	36 ± 2	94 ± 3
E	91 ± 3	17 ± 2	61 ± 4
SI	98 ± 2	15 ± 2	59 ± 2

[a] Expressed as percentage of proteins in the SFP product. [b] Expressed as percentage of solids with respect to the amount present in the seeds. [c] Expressed as percentage of proteins with respect to the proteins present in the seeds.

Protein Extraction. To find the optimal pH for protein extraction, protein extractability was determined as a function of pH (**Figure 1**). The extractability of the DM follows the expected pattern for a nondenatured meal (*51*, *52*): low extractability around the isoelectric point (pH 5) and an increase in extractability with increasing pH. However, the extraction of proteins at very high pH values is not recommended because under these conditions proteins could be chemically altered (*53*). Therefore, protein extraction for further experiments was carried out at pH 9. Protein concentration and further purification are reached by diafiltration of the extract yielding the SI. After this step, the protein content increased about 7% due to the removal of small compounds (**Table 3**).

The whole process developed integrates a series of steps: defatting, solvent washing, extraction at pH 9, diafiltration of the supernatant, and drying. **Table 3** summarizes the mass and protein yield of the isolation procedure for SFPs. As can be deduced from these data, removal of components other than CGA also occurs during dephenolization. The protein extract obtained at pH 9 already had a high protein content (91%). This content can be increased up to approximately 98% by membrane filtration. After the complete process, 60% of the total protein is recovered, which is similar to yields obtained previously (*7*, *18*, *23*, *25*, *54*). The 2S fraction, which has a high isoelectric point, is probably not fully recovered because of the high pH of extraction. The isolate has a CGA content lower than 0.01% and does not have the intense green color normally observed in the isolate produced by conventional alkali extraction followed by acid precipitation (*7*), but it is rather characterized by a light brown, creamy color. Furthermore, no denaturation occurred during the complete isolation procedure, as can be deduced from the DSC analysis of the SI (**Table 2**), since the enthalpy of denaturation per gram of protein is also the same as the one found for the proteins in the seed.

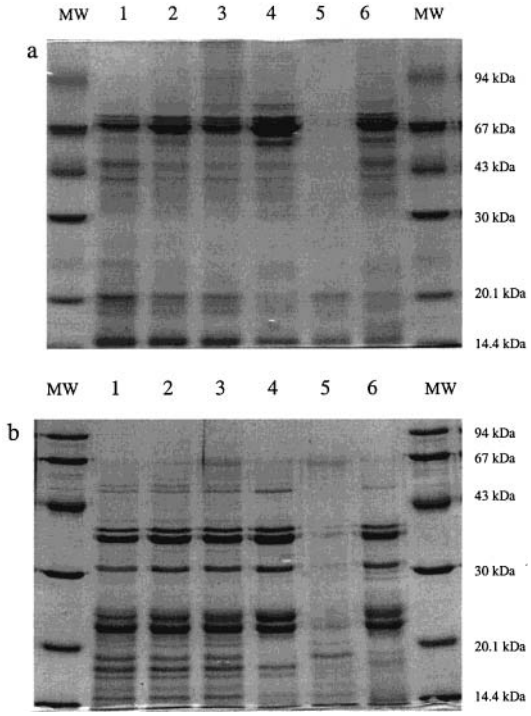

Figure 2. SDS–PAGE patterns of SFP products analyzed using 12% gels. (a) Without and (b) with reduction. Lane 1, SM; lane 2, DM; lane 3, DDM; lane 4, E; lane 5, P; and lane 6, SI. The molecular weights of marker proteins (MW lanes) are indicated.

Characterization of Sunflower Products. *Protein Characterization.* SDS−PAGE analysis under nonreducing (**Figure 2a**) and reducing (**Figure 2b**) conditions was performed to identify the protein composition of SFP and to investigate the effect of the isolation procedure on protein composition. In **Figure 2a**, two main groups of proteins can be distinguished: the group of high molecular weight (HMW) proteins consisting mainly of proteins with a molecular weight of about 60−70 kDa and LMW proteins with a molecular weight of less than 20 kDa. These proteins have been previously identified as 11S globulins and 2S albumins (*55*, *56*). The 11S fraction is reported to have a molecular weight of 300−350 kDa and to be composed of six subunits (*57*, *58*). Each subunit contains two disulfide-linked polypeptide chains (*50*). After it is reduced (**Figure 2b**), the HMW fraction appears to be split into polypeptides of approximately 40, 30, and 24 kDa, in agreement with the findings of Dalgaredondo et al. (*55*).

These findings indicate that this sunflower variety consists of 11S and 2S proteins. This is further confirmed by gel permeation chromatography. The protein present in the DDM and SI eluted into two major peaks (**Figure 3A**,**B**) corresponding to the 11S (peak I) and 2S (peak II) fraction, respectively, as confirmed by the SDS−PAGE analysis of the proteins present in the peaks (results not shown).

During defatting and dephenolizing, almost no protein disappeared (**Table 3**). However, protein extraction at pH 9 resulted in a loss of part of the 2S albumins, as shown in **Figure 3**. As can be seen in this figure, the ratio of peak II (2S) to peak I (11S) decreases upon extraction. This fact is confirmed by gel electrophoresis, showing that the pellet remaining after extraction mainly contains LMW proteins (**Figure 2a**, lane 5). The reduction of 2S albumin is probably due to the high isoelectric point of these proteins (*56*), which makes them less soluble at pH 9.

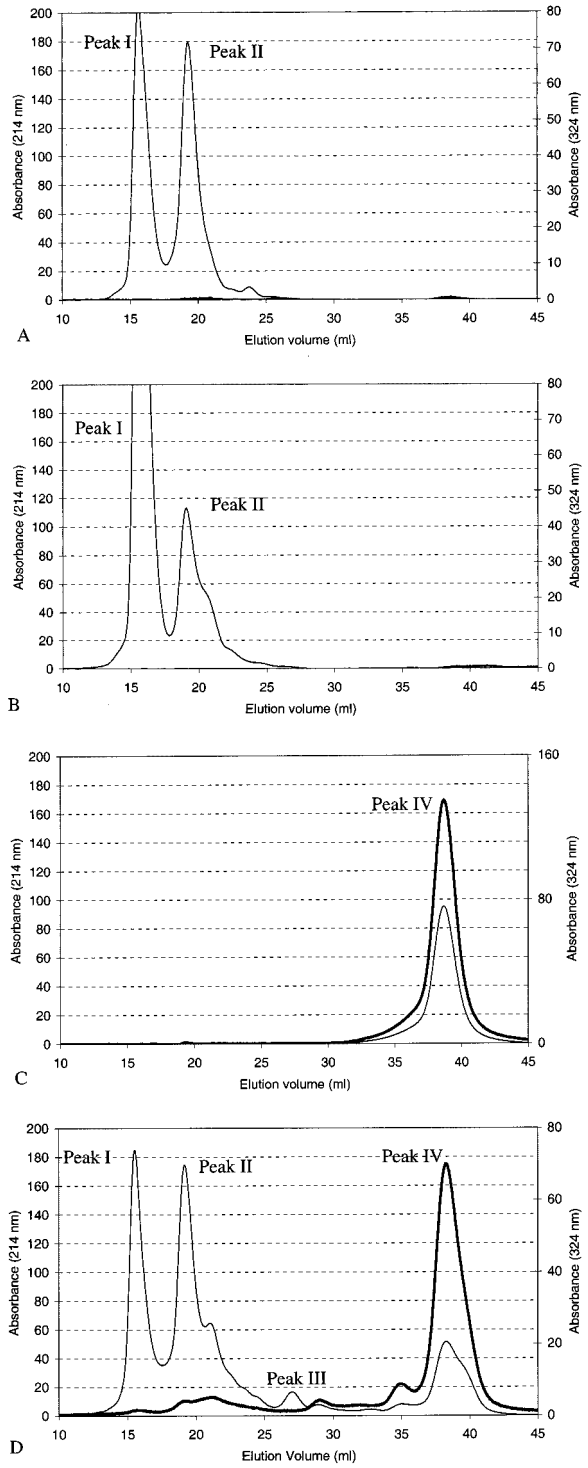

Figure 3. Chromatograms of SFPs monitored at 214 nm (—), and 324 nm (**—**); the absorbance is given in milliabsorbance units (mAU). Panel A, DDM; panel B, SI; panel C, pure CGA; panel D, SM.

Characterization of Carbohydrates. **Table 4** shows the total sugar content and molar neutral sugar composition of the mono-, oligo-, and polysaccharides present in the different sunflower products. From the total sugar content, it can be seen that as expected, most of the carbohydrates are removed during the production of the isolate. The carbohydrate composition of the SI, high amounts of arabinose, galactose, and uronic acid, is typical for pectic substances and strongly resembles that of a 0.05 M Na_2CO_3 extract of sunflower meal (*59*).

The dispersion of the freeze-dried SI in sodium acetate buffer, pH 5, and extensive washing resulted in liberation of arabinose

Table 4. Total Sugar Content and Molar Neutral Sugar Composition of the Mono-, Oligo-, and Polysaccharides Present in the Different Sunflower Products

		molar composition (%)					
	total sugar content[a]	Ara	Xyl	Man	Gal	Glc	UA
SM	10	16	6	7	11	46	14
DM	18	19	7	7	10	42	15
DDM	14	27	10	9	7	27	22
SI	2	29	6	9	21	11	27

[a] Expressed as weight percentage of each fraction. Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acids.

and galactose rich pectic material and lowered the total sugar content of the SI to 0.6% (w/v), which is relatively enriched in uronic acid (no further results shown). Incubation of the SI fraction with specific pectinolytic enzymes (polygalacturonase, pectin lyase, pectin methylesterase, rhanogalacturonase, rhamnogalacturonan acetyl esterase, *endo*-arabanase, *endo*-galactanase, *endo*-glucanase V, and combinations thereof) did not result in a further lowering of the total sugar content as compared to the addition of sodium acetate buffer alone. This points at either physical or chemical enzyme inaccessible pectic material rather than a specific covalently carbohydrate−protein complex.

Interaction of CGA with Proteins. To determine whether CGA is bound to the proteins, gel permeation chromatography was performed. Absorbance was monitored at 214 nm to detect proteins and at 324 nm to specifically monitor CGA. However, it should be emphasized that not only proteins but also CGA absorbs at 214 nm (**Figure 3C**).

In the chromatograms of DDM and SI (**Figure 3A**,**B**), no absorbance was measured at 324 nm, which is a clear indication that CGA has been removed efficiently. Subsequently, all 214 nm peaks in these chromatograms (denoted peak I and peak II) can be ascribed to the SFPs.

However, the 214 nm chromatogram of sunflower seed (**Figure 3D**) reveals two additional peaks. Peak III, which absorbs at 214 nm but not at 324 nm, can be attributed to small molecular weight material eluting at the total volume of the column (about 25 mL). Peak IV is most probably due to the presence of CGA. It has maximum absorbance at 324 nm, and it elutes at the same position as free CGA (**Figure 3C**). Furthermore, the spiking of DM with pure CGA showed that this peak can be attributed to free CGA. After the DM was spiked, the ratio of the total area of all peaks at 214 nm over those at 324 nm decreased, whereas it was constant when evaluated only for peak IV. Moreover, its position far behind the total volume of the column is in accordance with the observations that aromatic compounds interact with agarose- or dextran-based gel materials (*60*).

Many authors reported that CGA appeared mainly in the form of complexes or with proteins in sunflower products, either preferentially, with LMW proteins (*8*, *13*, *48*, *57*, *61*) or HMW protein (*4*), or nonpreferentially (*19*, *30*). Some of these authors detected, using gel permeation chromatography, peaks similar to the denoted peak IV. These peaks detected had their maximum absorbance at 324−328 nm, and also, their elution was retarded by the column. These peaks were interpreted as CGA−protein complexes rather than CGA. This interpretation was mainly based on the absorbance reduction at 280 nm upon dialysis. However, to our opinion, this reduction is due to the removal of CGA since this compound also absorbs at 280 nm. On the contrary, the absence of staining for protein in the polyacrylamide electrophoresis (results not shown) and the experiments described above confirms that peak IV solely consists of CGA. Our observations clearly show that most of the CGA elutes as free CGA at high elution volumes rather than as protein−CGA complexes.

Summarizing, when aqueous methanol is used for the removal of CGA from sunflower SM, a protein isolate free of CGA and consisting of nondenatured protein can be obtained. In addition, with the method used, the CGA does not form complexes with proteins.

ABBREVIATIONS USED

SFP, sunflower protein; FAO, Food and Agriculture Organization; CGA, chlorogenic acid; CA, caffeic acid; SM, seed meal; DM, defatted meal; DDM, defatted dephenolized meal; E, extract P, pellet; SI, sunflower isolate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DSC, differential scanning calorimetry; HMW, high molecular weight; LMW, low molecular weight.

LITERATURE CITED

(1) Miller, N.; Pretorius, E. Evaluation of the effect of processing on sunflower protein quality. *Food Chem.* **1985**, *17*, 65−70.
(2) Lusas, E. W. Sunflower seed protein. In *New Protein Foods*; Altschul, A. M., Wilcke, H. L., Eds.; Academic Press Inc.: Orlando, FL, 1985; pp 393−433.
(3) Sripad, G.; Narasinga Rao, M. S. Effect of methods to remove polyphenols from sunflower meal on the physicochemical properties of the proteins. *J. Agric. Food Chem.* **1987**, *35*, 962−967.
(4) Sastry, M. C. S.; Rao, M. S. N. Binding of chlorogenic acid by the isolated polyphenol-free 11S protein of sunflower (*Helianthus annuus*) seed. *J. Agric. Food Chem.* **1990**, *38*, 2103−2110.
(5) Mikolajczak, K. L.; Smith, C. R., Jr.; Wolff, I. A. Phenolic and sugar components of Armavirec variety sunflower (*Helianthus annuus*) seed meal. *J. Agric. Food Chem.* **1970**, *18*, 27−32.
(6) Sabir, M. A.; Sosulski, F. W.; Kernan, J. A. Phenolic constituents in sunflower flour. *J. Agric. Food Chem.* **1974**, *22*, 572−574.
(7) Lawhon, J. T.; Glass, R. W.; Manak, L. J.; Lusas, E. W. White-colored protein isolate from sunflower: processes and products. *Food Technol.* **1982**, *36*, 86−87.
(8) Venktesh, A.; Prakash, V. Low molecular weight proteins from sunflower (*Helianthus annuus L.*) seed: effect of acidic butanol treatment on the physicochemical properties. *J. Agric. Food Chem.* **1993**, *41*, 193−198.
(9) Pomenta, J. V.; Burns, E. E. Factors affecting chlorogenic, quinic and caffeic acid levels in sunflower kernels. *J. Food Sci.* **1971**, *36*, 490−492.
(10) Cater, C. M.; Gheyasuddin, S.; Mattil, K. F. The effect of chlorogenic, quinic, and caffeic acids on the solubility and color of protein isolates, especially from sunflower seed. *Cereal Chem.* **1972**, *49*, 508−514.
(11) Sodini, G.; Canella, M. Acidic Butanol Removal of Color-Forming Phenols from Sunflower Meal. *J. Agric. Food Chem.* **1977**, *25*, 822−825.
(12) Saeed, M.; Cheryan, M. Sunflower protein concentrates and isolates low in polyphenols and phytate. *J. Food Sci.* **1988**, *53*, 1127−1143.
(13) Prasad, D. T. Proteins of the phenolic extracted sunflower meal: I. Simple method for removal of polyphenolic components and characteristics of salt soluble proteins. *Lebensm.-Wiss. Technol.* **1990**, *23*, 229−235.
(14) Venktesh, A.; Prakash, V. Functional properties of the total proteins of sunflower (*Helianthus annuus* L.) seed: effect of physical and chemical treatments. *J. Agric. Food Chem.* **1993**, *41*, 18−23.

(15) Regitano d'Arce, M. A.; Gutierrez, E. M.; Lima, U. d. A. Sunflower seed protein concentrates and isolates obtention from ethanol oil extraction meals (technical note). *Arch. Latinoam. Nutr.* **1994**, *44*, 33−35.

(16) Sanchez, A. C.; Burgos, J. Thermal Gelation of Sunflower Proteins. In *Food Macromolecules and Colloids*; Dickinson, E., Lorient, D., Eds.; Royal Society of Chemistry: Cambridge, United Kingdom, 1995; pp 426−430.

(17) O'Connor, D. E. Preparing light-colored protein isolate from sunflower meal by acid washing prior to alkaline extraction. United States Patent 3,586,662, 1971.

(18) Hagenmaier, R. D. Aqueous processing of full-fat sunflower seeds: yields of oil and protein. *J. Am. Oil Chem. Soc.* **1974**, *51*, 470−471.

(19) Rahma, E. H.; Narasinga Rao, M. S. Isolation and characterization of the major protein fraction of sunflower seeds. *J. Agric. Food Chem.* **1981**, *29*, 518−521.

(20) Pearce, R. J. Preparation of protein isolate from sunflower seed. United States Patent 4,435,319, 1984.

(21) Shamanthaka Sastry, M. C.; Subramanian, N. Preliminary studies on processing of sunflower seed to obtain edible protein concentrates. *J. Am. Oil Chem. Soc.* **1984**, *61*, 1039−1042.

(22) Shamanthaka Sastry, M. C.; Narasinga Rao, M. S. Binding of chlorogenic acid by the isolated polyphenol-free 11S protein of sunflower (*Helianthus annuus*) seed. *J. Agric. Food Chem.* **1990**, *38*, 2103−2110.

(23) O'Connor, D. E. Preparing light-coloured protein isolate from sunflower meal by alkali extraction under an inert gas blanket followed by membrane ultrafiltration. United States Patent 3,622,556, 1971.

(24) Petit, L.; Davin, A.; Gueguen, J. Purified sunflower seed protein isolates. United States Patent 4,174,313, 1979.

(25) Nuzzolo, C.; Vignola, R.; Groggia, A. Method for preparing a proteinic isolate from sunflowerseed meal using aluminum salts. United States Patent 4,212,799, 1980.

(26) Bau, H. M.; Mohtadi Nia, D. J.; Mejean, L.; Debry, G. Preparation of colorless sunflower protein products: Effect of processing on physicochemical and nutritional properties. *J. Am. Oil Chem. Soc.* **1983**, *60*, 1141−1148.

(27) Gheyasuddin, S.; Cater, C. M.; Mattil, K. F. Preparation of a colourless sunflower protein isolate. *Food Technol.* **1970**, *24*, 242−243.

(28) Sosulski, F. W.; McCleary, C. W.; Soliman, F. S. Diffusion extraction of chlorogenic acid from sunflower kernels. *J. Food Sci.* **1972**, *37*, 253−256.

(29) Fan, T. Y.; Sosulski, F. W.; Hamon, N. W. New techniques for preparation of improved sunflower protein concentrates. *Cereal Chem.* **1976**, *53*, 118−125.

(30) Rahma, E. H.; Narasinga Rao, M. S. Characterization of sunflower proteins. *J. Food Sci.* **1979**, 579−582.

(31) Rahma, E. H.; Narasinga Rao, M. S. Removal of polyphenols from sunflower meal by various solvents: effects on functional properties. *J. Food Sci.* **1981**, *46*, 1521−1522.

(32) Raymond, J.; Dalgalarrondo, M.; Azanda, J. L.; Ducastaing, A. Preparation of protein isolates from sunflower cakes. *Rev. Fr. Corps Gras* **1984**, *6*, 233−242.

(33) Tranchino, L.; Costantino, R.; Sodini, G. Food grade oilseed protein processing: sunflower and rapeseed. *Qual. Plant. Plant Foods Hum. Nutr.* **1983**, *32*, 305−334.

(34) Vermeesch, G.; Briffaud, J.; Joyeux, J. Sunflower proteins in human food. *Rev. Fr. Corps Gras* **1987**, *7*−*8*, 333−344.

(35) Lustig, B.; Fink, A. L. The thermal denaturation of ribonuclease A in aqueous methanol solvents. *Biochim. Biophys. Acta* **1992**, *1119*, 205−210.

(36) Srinivasulu, S.; Rao, A. G. A. Structure and kinetic thermal stability studies of the interaction of monohydric alcohols with lipoxygenase 1 from soybeans (*glycine max*). *J. Agric. Food Chem.* **1995**, *43*, 562−567.

(37) Bakhuni, V. Alcohol-induced molten globule intermediates of proteins: Are they really folding intermediates or off pathway products? *Arch. Biochem. Biophys.* **1998**, *357*, 274−284.

(38) Grinberg, V. Y.; Grinberg, N. Y.; Burova, T. V.; Dalgalarrondo, J.; Haertlé, T. Ethanol-induced conformational transitions in holo-a-lactalbumin: Spectral and calorimetric studies. *Biopolymers* **1998**, *46*, 253−265.

(39) Berot, S.; Briffaud, J. Parameters for obtaining concentrates from rapeseed and sunflower meal. *Qual. Plant. Plant Foods Hum. Nutr.* **1983**, *33*, 237−242.

(40) Sanchez, A. C.; Burgos, J. Gelation of sunflower globulin hydrolysates: Rheological and calorimetric studies. *J. Agric. Food Chem.* **1997**, *45*, 2407−2412.

(41) *Approved Methods of the American Association of Cereal Chemists*, 9th ed. The American Association of Cereal Chemists Inc.: St. Paul, MN, 1995.

(42) Englyst, H. N.; Cummings, J. H. Simplified method for the measurement of total nonstarch polysaccharides by gas−liquid chromatography of constituent sugars as alditol acetates. *Analyst* **1984**, *109*, 938−942.

(43) Thibault, J.-F. Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphenyl. *Lebensm.-Wiss. Technol.* **1979**, *12*, 247−251.

(44) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 681−685.

(45) Moores, R. G.; McDermott, D. L.; Wood, T. R. Determination of chlorogenic acid in coffee. *Anal. Chem.* **1948**, *20*, 620−624.

(46) Milic, B.; Stojanovic, S.; Vucurevic, N.; Turcic, M. Chorogenic and quinic acids in sunflower meal. *J. Sci. Food Agric.* **1968**, *19*, 108−113.

(47) Rossi, M.; Peri, C.; Riva, M. Protein concentrate production from sunflower cakes: II. Simultaneous extraction of oil and clorogenic acid. *Riv. Ital. Sostanze Grasse* **1980**, *57*, 509−513.

(48) Sabir, M. A.; Sosulski, F. W.; Finlayson, A. J. Chlorogenic acid−protein interactions in sunflower. *J. Agric. Food Chem.* **1974**, *22*, 575−578.

(49) Tolstoguzov, V. B. Some physicochemical aspects of protein processing into foodstuffs. *Food Hydrocolloids* **1988**, *2*, 339−370.

(50) Grinberg, V.; Danilenko, A. N.; Burova, T. V.; Tolstoguzov, V. B. Conformational stability of 11S globulins from seeds. *J. Sci. Food Agric.* **1989**, *49*, 235−248.

(51) Mattil, K. F. The functional requirements of proteins for foods. *J. Am. Oil Chem. Soc.* **1971**, *48*, 477−480.

(52) Clark, S. P.; Wan, P. J.; Matlock, S. W. Pilot plant production of sunflower seed flour. *J. Am. Oil Chem. Soc.* **1980**, *57*, 267−279.

(53) Provansal, M. M. P.; Cuq, J. L. A.; Cheftel, J. C. Chemical and nutritional modifications of sunflower proteins due to alkaline processing. Formation of amino acid cross-links and isomerization of lysine residues. *J. Agric. Food Chem.* **1975**, *23*, 938−943.

(54) Castor Normandin, F.; Ducastaing, A.; Prevot, A.; Raymond, J. Preparation of protein isolates from sunflower cakes. *Rev. Fr. Corps Gras* **1984**, *7*−*8*, 293−299.

(55) Dalgalarrondo, M.; Raymond, J.; Azanza, J. L. Sunflower seed protein: size and charge heterogeneity in subunits of the globulin fraction. *Biochimie* **1985**, *67*, 629−632.

(56) Kortt, A. A.; Caldwell, J. B. Low molecular weight albumins from sunflower seed: Identification of a methionine-rich albumin. *Phytochemistry* **1990**, *29*, 2805−2810.

(57) Sabir, M. A.; Sosulski, F. W.; MacKenzie, S. L. Gel chromatography of sunflower proteins. *J. Agric. Food Chem.* **1973**, *21*, 988−993.

(58) Schwenke, K. D.; Paehtz, W.; Linow, K. J.; Raab, B.; Schultz, M. On seed proteins. XI. Purification, chemical composition, and some physicochemical properties of the 11 S globulin (Helianthinin) in sunflower seed. *Nahrung* **1979**, *23*, 241−254.

(59) Dusterhoft, E. M.; Engels, F. M.; Voragen, A. G. J. Nonstarch polysacchrides from sunflower (*Helianthus annuus*) meal and

palm-kernel (*Elaeis guineensis*) meal. I. Preparation of cell wall material and extraction of polysaccharide fraction. *J. Sci. Food Agric.* **1991**, *55*, 411−422.

(60) Haslam, E. Molecular recognition-Phenols and polyphenols. In *Practical Polyphenolics. From Structure to Molecular Recognition and Physiological Action*; Haslam, E., Ed.; Cambridge University Press: Cambridge, United Kingdom, 1998; pp 138−177.

(61) Kabirullah, M.; Wills, R. B. H. Characterization of sunflower protein. *J. Agric. Food Chem.* **1983**, *31*, 953−956.

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