Physicochemical Properties of 2S Albumins and the Corresponding Protein Isolate from Sunflower (*Helianthus annuus*)

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ABSTRACT: Sunflower albumins (SFAs) are a diverse group of proteins present in sunflower isolates, with a sedimentation coefficient of approximately 2S. This research presents a detailed study of the influence of pH on the structure and solubility of SFAs. The effect of temperature on the structure of SFAs was also studied. Furthermore, the solubility of a sunflower isolate (SI) was studied and discussed in terms of its main protein components (SFAs and helianthinin). The native structure of SFAs revealed to be very stable against pH changes (pH 3.0 to 9.0) and heat treatment (>100 °C), and their solubility was only marginally affected by pH and ionic strength. The solubility of the sunflower isolate as a function of pH seems to be dominated by that of helianthinin: SI (I = 30 mM) showed a U-shaped solubility curve with a minimum between pH 4.0 and pH 6.0.

Keywords: *Helianthus annuus*, sunflower, albumin, stability, denaturation

Introduction

Sunflower seeds are used in the food industry as a source of oil. The main by-product of the oil extraction process is sunflower meal (SFM), which has high protein content (40% to 50%) (Salunkhe and others 1992). This high protein content makes SFM an attractive source for the isolation of proteins. 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 (Gassmann 1983; Miller and Pretorius 1985). However, the suitability for food applications of the SFM proteins depends mainly on the oil extraction method. Because of this process, the proteins may be denatured to a large extent, resulting in a SFM with high content of insoluble proteins (Parrado and others 1993). 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). The interaction with phenolic compounds can affect sunflower protein in several ways, such as reducing protein digestibility and functionality, prolonging or shortening its storage life and stability, and altering its organoleptic properties (Sripad and Rao 1987; Sastry and Rao 1990). Furthermore, the presence of CGA results in a dark coloration of sunflower protein products. Removal of phenolic compounds is, therefore, one of the main problems concerning the production of sunflower protein products (Milic and others 1968; Gassmann 1983; Tranchino and others 1983; Venktesh and Prakash 1993).

The preparation of a sunflower isolate, free of phenolic compounds and non-denatured, has been recently described (González-Pérez and others 2002). Two main groups of proteins are present in this sunflower isolate (SI): helianthinin and sunflower albumins (SFAs). Several studies have shown that these proteins are the 2 major classes of globular proteins present in sunflower seeds (Youle and Huang 1981; Dalgalarrondo and others 1984; Mazhar and others 1998; Anisimova and others 2002). SFAs are a diverse group of proteins, usually soluble in water, with a sedimentation coefficient of approximately 2S, of which some are rich in cysteine. They have been reported to be basic proteins (isoelectric pH [pI] of approximately 8.8) and to have molecular weights (MWs) ranging from about 10 to 18 kDa (Kortt and Caldwell 1990; Anisimova and others 1995; Raymond and others 1995; Popineau and others 1998). In contrast to 2S albumins from other seed species (that is, Brazil nut, oilseed rape, mustard seed, and so forth), which are consisting of 2 chains linked by disulfide bonds, SFAs consist of a single polypeptide chain (Allen and others 1987; Anisimova and others 1995; Shewry and Pandya 1999). SFAs are polymorphic, and 8 to 13 individual SFA proteins have been separated by reverse-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, the total number of components may be larger (Kortt and Caldwell 1990; Anisimova and others 1995). The levels at which these components are present vary widely between different genotypes (Anisimova and others 1995, 2002). The amino acid sequences of 2 SFA proteins are available: (1) the so-called 2S albumin storage protein (HAG5) consisting of 134 amino acids, having a MW of 15777 Da and a theoretical isoelectric pH (pI) of 8.69; and (2) a methionine-rich 2S protein consisting of 103 amino acids, having a MW of 12133 Da and theoretical pI of 5.91 (Allen and others 1987; Kortt and others 1991; Swiss-prot p15461; Swiss-prot p23110). The latter protein is called SFA8 based on its order of elution on RP-HPLC (Kortt and Caldwell 1990).

Despite the research performed in the past decades, not much is known about the structure and behavior of SFAs in solution. Heat treatments (that is, toasting) and treatment at acidic pH values (for

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example, during large-scale sunflower isolate preparation) are commonly used in food industry, but may alter protein structure. These structural modifications may easily result in changes in the functional properties of a protein, for example, its solubility, which is a prerequisite for various functional properties such as emulsion and foam properties (Kinsella 1979). Therefore, knowledge on protein structure and conformational stability at various conditions is important, in connection with solubility, during protein isolation and subsequent application in food products.

This research presents a detailed study of the influence of pH on the structure and solubility of SFAs. The effect of temperature on protein structure is also studied. Furthermore, the solubility of the sunflower isolate is studied and discussed in terms of its main protein components.

Materials and Methods

Protein isolation

Defatting and dephenolising. Dehulled "Mycogen Brand" sunflower seeds, purchased from H. Ch. Schobbers B.V. (Echt, The Netherlands), were milled in a laboratory grinder (Janke and Kunkel GmbH, Staufen, Germany) for 3 min. High temperatures were avoided by cooling the grinder periodically with liquid nitrogen. The resulting meal was defatted with hexane and dephenolized by cold extraction of the phenolic compounds with 80% (v/v) methanol as described previously (González-Pérez and others 2002). This procedure yields the defatted dephenolized meal (DDM).

Sunflower isolate preparation. The DDM obtained was suspended in water (2% [w/v]) and stirred for 30 min, while keeping the pH at 9.0 by addition of 1 *N* NaOH. Soluble protein was recovered after centrifugation (30000 × *g*, 20 min, 20 °C). The pellet was re-extracted (similar conditions), and the 2 supernatants were combined to yield the extract. 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, Mass., U.S.A.). The retentate obtained was subsequently freeze-dried and denoted SI.

Sunflower albumins (SFAs) isolation. The DDM obtained was suspended in water (2% [w/v]) and stirred for 2 h while keeping the pH at 5.0 by addition of small volumes of 1 *N* HCl. Continuous centrifugation was carried out in a vertical centrifuge type V30-O/703 (Heine; GFT Trenntechnik, Viersen, Germany) at the maximum speed of 3500 μm. Filter cloths (mesh size 1 mm) were purchased at Lampe technical textiles BV (Sneek, The Netherlands). The pellet was re-extracted at similar conditions (2% [w/v] suspension, pH 5.0) and the 2 supernatants were combined. Ammonium sulfate was added to the total supernatant up to 90% saturation, and the mixture was stored for 30 min at 4 °C. After centrifugation (10000 × *g*, 20 min, 4 °C), the supernatant was discarded and the pellet was washed (2% [w/v]) once with an ammonium sulfate solution (90% saturation) at 4 °C. After centrifugation (10000 × *g*, 20 min, 4 °C), the final pellet was dissolved in distilled water and desalted by diafiltration using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a MW cut-off of 3 kDa (A/G Technology Corp., Needham, Mass., U.S.A.) until the conductivity of the retentate remained constant (55 μS/cm). The retentate obtained was freeze-dried to yield the SFAs extract. Further purification was performed by gel permeation chromatography. The SFAs extract was dissolved (1% [w/v]) in 30 m*M* sodium phosphate buffer (pH 8.0), and 150 mL of the solution was applied, after filtration over a 0.45-μm filter, on a Superdex 200 column (68 × 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The column was eluted with the same buffer at a linear flow rate of 30 cm/h. The 2nd peak, as observed from the absorbance at 280 nm, was collected, diafiltrated using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a MW cut-off of 3 kDa (A/G Technology Corp., Needham, Mass., U.S.A.) until the conductivity of the retentate remained constant, and freeze-dried to produce purified SFAs.

Determination of protein solubility. Protein solubility experiments were performed with SI and SFAs. The proteins were dispersed to a final concentration of 4.0 mg/mL in water and the pH adjusted to 8.5 by addition of small amounts of NaOH solutions. The ionic strength was adjusted to 0.03 *M* or 0.25 *M* by adding NaCl. The pH of the protein solutions was lowered by adding various amounts of HCl solutions to obtain final pH values ranging from 2.0 to 8.5 with 0.5 pH unit intervals. The samples were stored for about 2 h at room temperature. Next, the samples were centrifuged for 15 min at 15800 × *g* at 20 °C. The protein concentration of the supernatants was determined in triplicate using the Bradford method (Bradford 1976) with bovine serum albumin (BSA) as a standard. Solubility was expressed as proportion (%) of the amount of protein dissolved at pH 8.5. All the solubility experiments were performed at least in duplicate.

Protein concentration as estimated by Bradford (Bradford 1976) and the Dumas combustion method were compared and found to be similar. For the latter method, a NA 2100 nitrogen analyzer (CE instruments, Milan, Italy) was used according to the instructions of the manufacturer.

Analysis

Protein content. Protein content (N × 5.55) of the SI and SFAs isolate was determined by the Kjeldahl method, AACC 46-12 (AACC 1995).

Protein size and composition. Protein size and composition was estimated by analytical gel permeation chromatography and gel electrophoresis.

Gel permeation chromatography. Gel permeation chromatography was performed using an Äkta Explorer System (Amersham, Pharmacia Biotech). Protein samples (0.2 to 2.0 mg/mL) were dissolved at room temperature in 50 m*M* sodium phosphate buffer, pH 6.9, containing 0.25 *M* NaCl. After filtration over a 0.2-μm filter, the samples were applied (0.2 mL) on a Superdex 200 HR 10/30 (30 × 1 cm) column and eluted with the same buffer at a flow rate of 0.5 mL/min at room temperature. The column was calibrated using markers ranging from 13 to 2000 kDa (Amersham, Pharmacia Biotech): Ribonuclease A (13700 Da), ovalbumin (43000 Da), BSA (67000 Da), aldolase (158000 Da), catalase (232000 Da), ferritin (440000 Da), and blue dextran (2000000 Da). The absorbance of the eluate was monitored at 214 and 280 nm.

Gel electrophoresis. SDS-PAGE was performed according to the method of Schägger and von Jagow (Schagger and von Jagow 1987) on a Mini-PROTEAN II electrophoresis system (Bio-Rad, Veenendaal, The Netherlands), following the instruction of the manufacturer. Protein samples of 13 μg were dissolved in sample buffer, and applied to precast 16.5% Tris-tricine gels (Bio-Rad). After electrophoresis the gels were stained with Coomassie Brilliant Blue. Protein markers used ranged from 3.5 to 26.6 kDa (Bio-Rad): bovine insulin (3496 Da), aprotinin (6500 Da), lysozyme (14400 Da), myoglobin (16950 Da), and triosephosphate isomerase (26625 Da).

Differential scanning calorimetry. Calorimetric studies were performed using a VP-DSC MicroCalorimeter (MicroCal Inc., Northhampton, Mass., U.S.A.). Thermograms were recorded from 20 °C to 130 °C with a heating rate of 1 °C/min. Experiments were performed with SFAs at protein concentrations of 1.0 to 3.0 mg/mL at

several pH values: pH 3.0 (10 m*M* sodium phosphate buffer), pH 6.2 (10 m*M* sodium phosphate buffer), pH 7.0 (10 m*M* sodium phosphate buffer), and pH 9.0 (10 m*M* sodium borate buffer). The final ionic strength of the buffers was adjusted to 30 m*M* by adding NaCl. Protein concentration of the solutions was estimated by absorbance measurement at 280 nm (ϵ = 0.78 mL/cm mg). All measurements were carried out at least in duplicate.

Circular dichroism spectroscopy (Far-UV). Far-UV circular dichroism (CD) spectra of SFAs samples were recorded at 20 °C, 110 °C, and at 20 °C after heat treatment at 110 °C, as averages of 10 spectra on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) at several pH values: pH 3.0 (10 m*M* sodium phosphate buffer), pH 6.2 (10 m*M* sodium phosphate buffer), pH 7.0 (10 m*M* sodium phosphate buffer), and pH 9.0 (10 m*M* sodium borate buffer). The final ionic strength of the buffers was adjusted to 30 m*M* by adding NaF. Quartz cells with an optical path length of 1 mm and 0.2 mm with protein concentrations of approximately 0.1 mg/mL and 0.04 mg/mL, respectively, were used. The scan range was 180 to 260 nm, the scan speed was 100 nm/min, the data interval was 0.2 nm, the bandwidth 1.0 nm, the sensitivity was 20 millidegrees and the response time 0.125 s. Spectra were corrected by subtracting the spectrum of a protein-free sample obtained under identical conditions. Noise reduction was applied using the Jasco software. The spectra were analyzed from 240 to 190 nm to calculate the secondary structure content of the protein using a nonlinear regression procedure as described in detail by Pots and coworkers (Pots and others 1998).

Changes in thermal stability of the secondary structure of proteins were also monitored by measuring the ellipticity at 200 nm as a function of temperature at a heating rate of 1 °C/min.

Mass spectrometry (MALDI-TOF). Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Voyager-DE^{TM}RP Mass spectrometer (PerSeptive Biosystems Inc., Framingham, Mass., U.S.A.) equipped with UV nitrogen laser (337 nm). The instrument was operated in linear mode. Spectra were obtained in positive ion mode using an acceleration voltage of 25 kV and a delay time of 400 ns. The samples (1 mg/mL) were dissolved in a 20 m*M* sodium phosphate buffer (pH 7.0) with and without addition of 30 m*M* dithiothreitol for the reducing and nonreducing conditions, respectively. Aliquots (1 μL) of the protein solutions were mixed with 9 μL matrix solution. The matrix solution consisted of sinapinic acid (10 mg/mL) in 50% (v/v) acetonitrile containing 0.3% (v/v) trifluoroacetic acid. The final mixtures were loaded on a welled plate and allowed to dry. All samples were analyzed at least in triplicate.

Results and Discussion

Protein composition

The protein contents of the SI (that consists of SFAs and helianthinin) and of the purified SFAs were both above 95%. The purified SFAs preparation is virtually free of phenolic compounds (<0.01%) as sunflower defatted dephenolized meal was used as starting purification material (González-Pérez and others 2002). Tricine SDS-PAGE of SFAs shows 2 main bands with approximate MWs of 12 and 15 kDa (Figure 1a). Besides these 2 bands, other minor ones may be observed, as expected from the heterogeneous composition of SFAs. However, mass spectrometry confirmed the presence of a 12.117 Da protein, but no peak could be detected at 15 kDa (no further data shown). The latter may be because of the poor ionization of 15 kDa protein. Gel filtration chromatography (pH 6.9) of the purified SFAs preparation showed 1 peak (Figure 1b), with an elution volume of 17.0 ± 0.2 mL, with a small shoulder at about 19 mL. Calibration of the column allowed the apparent MW to be estimated at 14 kDa.

Protein solubility

Because protein solubility is a prerequisite for functional application of proteins in foods, the effects of pH and ionic strength (*I*) on protein solubility were studied in the pH range 2.0 to 8.5, at *I* = 30 m*M* and I = 250 m*M*. The solubility of purified helianthinin (González-Pérez and others 2004) has been incorporated to discuss the solubility of sunflower isolate in relation to that of SFAs and helianthinin. The solubilities of the various sunflower protein preparations as a function of pH are shown in Figure 2. SFAs remained soluble independently of pH and ionic strength. At low ionic strength (*I* = 30 m*M*) helianthinin shows a bell-shaped curve with a minimum at pH 4.0 to 5.5 (Figure 2a), its isoelectric pH. At high ionic strength (*I* = 250 m*M*), helianthinin is almost insoluble at pH < 5.0 (Figure 2b). A similar trend can be seen for SI (Figure 2). Two pH regions can be distinguished at low ionic strength: At pH <5.5, the solubility of SI is higher than that of helianthinin, whereas at pH values between 5.5 and 7.0, the solubility of helianthinin is higher than that of the SI. At higher ionic strength, the region in which the solubility of helianthinin is higher than that of SI is reduced (Figure 2b). This indicates that electrostatic interactions between SFAs and helianthinin (which have opposite net charges at pH values between 5.5 and 7.0) may play a role in the reduction of solubility of

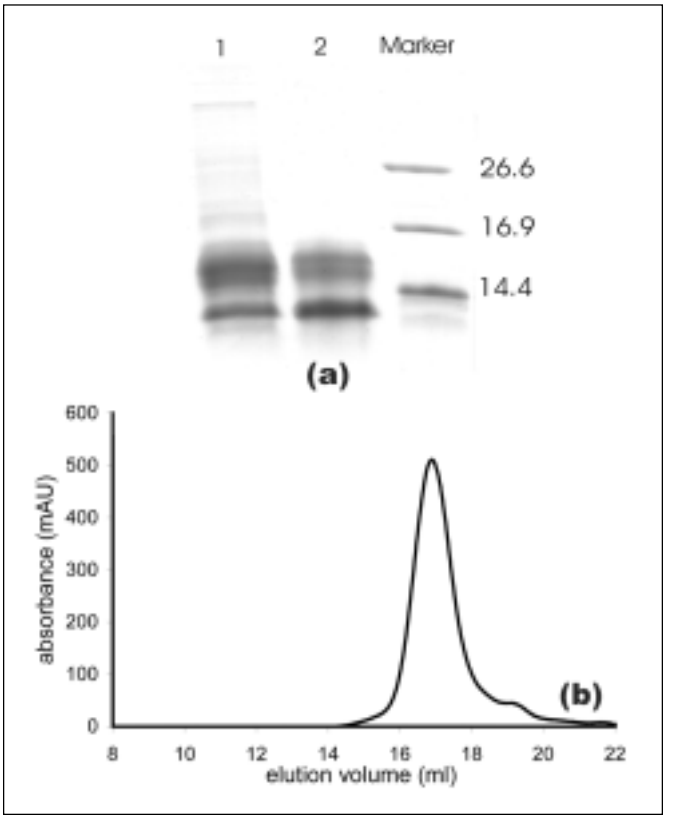

Figure 1—(a) Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of sunflower albumins (SFAs). Lane 1, SFAs extract; Lane 2, purified SFAs; Lane 3, marker. Only the relevant part of the gel is shown. (b) Chromatogram of SFAs at pH 6.9. The absorbance is monitored at 214 nm and is given in milliabsorbance units (mAU).

SI. However, the solubility of the sunflower isolate as a function of pH seems to be governed by helianthinin.

These results are in agreement with previous publications dealing with the solubility of various sunflower protein products (Gheyasuddin and others 1970; Mattil 1971; Sosulski and Fleming 1977; Canella 1978; Rossi and others 1985; Vermeesch and others 1987). However, Canella and coworkers (Canella and others 1985) reported minimum solubility of SFAs at pH 5.0. This divergence is probably because of the different composition of the albumin fraction and to possible contamination of the preparation with helianthinin, as could be inferred from the pH of minimum solubility. The decreased solubility of helianthinin at pH 3.0 (I = 30 mM), which could be attributed to denaturation and dissociation of the protein (González-Pérez and others 2004), is also observed for SI. A similar trend has been found by several authors (Gheyasuddin and others 1970; Mattil 1971; Cater and others 1972; Canella and others 1985). SI is estimated to contain about 13% to 25% SFAs according to the intensity of the bands in gel electrophoresis and to the area of the peaks as observed by GPC (no further results shown). The SFAs content of the SI isolate explains the lower protein solubility (10% to 25%) of helianthinin at pH <5.5 (I = 30 mM) and at pH <6.5 (I = 250 mM). The higher solubility of helianthinin in comparison with SI in the pH region of about 6.0 to 7.0 might be because of coprecipitation. This phenomenon has been previously shown to occur by Canella and coworkers (Canella and others 1985).

Conformational stability of sunflower albumins

Secondary structure at various pH values. Far-UV CD spectra of SFAs were recorded at pH 3.0, 6.2, 7.0, and 9.0 (I = 30) at 20 °C (Figure 3). The far-UV spectra are almost identical at all the pH values studied (Figure 3). The characteristic features are 2 minima about 209 and 222 nm, and a zero crossing of about 200 nm. Using curve-fitting procedures, the secondary structure content of SFAs was estimated. These estimations revealed that SFAs contain similar amounts (32%) of a-helical, b-sheet, and nonstructured elements.

Although the isolated SFAs consisted of at least 2 proteins according to their MW, and therefore no conclusive results can be deduced from the far-UV CD spectra, the similarity of the spectra of SFAs to those found for isolated SFA 8 in the research of Pandya and others (1999) is high. Both show a maximum at about 190 nm and minima close to 209 and 222 nm. Furthermore, our estimation in the amount of a-helical structure (32%) coincides with that of these authors (30%). Far-UV CD spectra for 2S albumins from rapeseed seed showed similar patterns (Krzyzaniak and others 1998).

Structure of SFAs as a function of temperature. Differential scanning calorimetry (DSC) thermograms of SFAs showed denaturation temperatures far above 100 °C, indicating that SFAs are very thermoresistant (Figure 4). The shape of the peaks was pH dependent. The peaks were sharp at pH 7.0 (denaturation temperature, T_d~=118 °C) and pH 9.0 (T_d[a] 107 °C), and broad at pH 3.0 and pH 6.2 (T_d~112 °C). The shape variation in the DSC peaks with pH may be caused by a change in the cooperativity in the unfolding of the protein and by possible changes in its aggregation behavior.

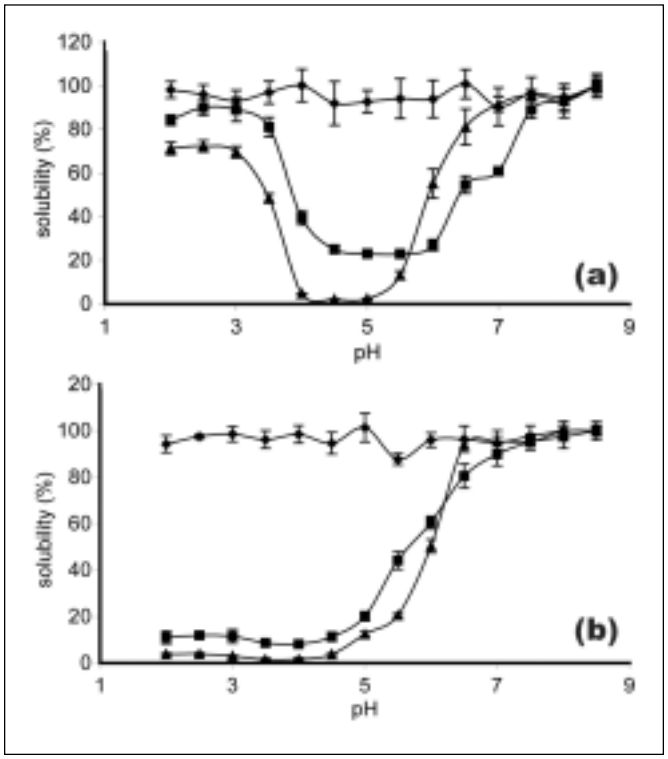

Figure 2—pH-dependent solubility profiles of helianthinin (González-Pérez and others 2004; ▲) sunflower albumins (SFAs) (◆), and sunflower isolate (SI) (■) at I = 30 mM (a) and I = 250 mM (b).

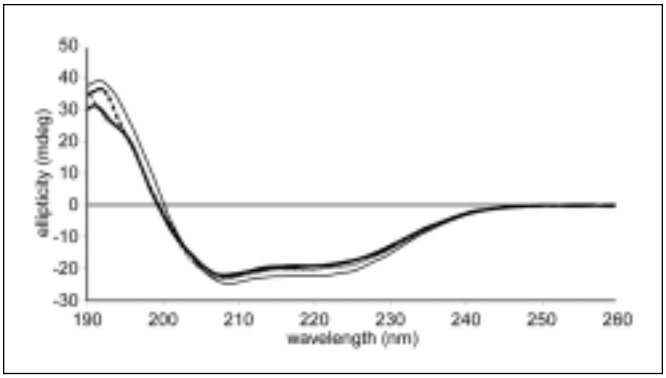

Figure 3—Far-UV circular dichroism (CD) spectra of sunflower albumins (SFAs) at pH 3.0 (dashed line), pH 6.2 (thick-dashed line), pH 7.0 (thin solid line), and 9.0 (thick solid line) at I = 30 mM.

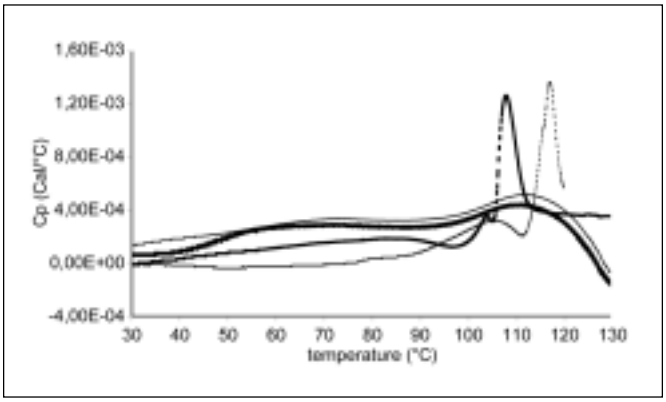

Figure 4—Differential scanning calorimetry (DSC) thermograms of sunflower albumins (SFAs) at pH 3.0 (thin solid line), pH 6.0 (thick solid line), pH 7.0 (dashed line), and pH 9.0 (thick-dashed line). For all samples, I = 10 mM.

Although DSC scans showed a good repeatability, calculation of the thermodynamic data for SFAs was impaired by the difficulty to draw reasonable baselines, and therefore, no enthalpy values are shown.

Figure 5a shows the far-UV CD spectra of SFAs at pH 7.0 (I = 30 mM) at 20 °C, 110 °C, and 20 °C after heating at 110 °C. To monitor changes in secondary structure, far-UV CD temperature scans were recorded at 200 nm from 20 °C to 110 °C (Figure 5b). In agreement with the DSC results, far-UV CD temperature scans showed only minor changes in the ellipticity between 20 °C and 110 °C (Figure 5b). Higher temperatures could not be tested because of limitation of the apparatus. In contrast to the DSC experiments, the thermal unfolding of the SFAs in the far-UV CD experiments seems to be partially reversible (Figure 5a). This is most likely due to the lower concentration used in the far-UV CD experiments compared with that in the DSC experiments.

SFAs revealed to be a group of proteins with a high conformational stability with respect to both pH and heat treatments. DSC as well as far-UV CD temperature scans revealed denaturation temperatures far above 100 °C. The data presented here are consistent with previous studies with a single sunflower albumin, SFA 8, in which the far-UV CD spectra of the protein did not vary over the pH range 2.0 to 10.0 or when heated to 90 °C (Pandya and others 1999). In the present research, however, no changes in secondary structure were observed at temperatures below 100 °C. The latter authors demonstrated the important role of disulfide bonds in maintaining the stability of the protein native fold. Molecular modeling studies predict that SFA8 has a compact structure with hydrophobic residues clustered to form a hydrophobic interface (Pandya and others 2000). This high stability seems to be a common feature of 2S seed proteins as 2S proteins from rapeseed were also found to be very stable (Muren and others 1996; Folawiyo and Owusu Apenten 1997; Krzyzaniak and others 1998).

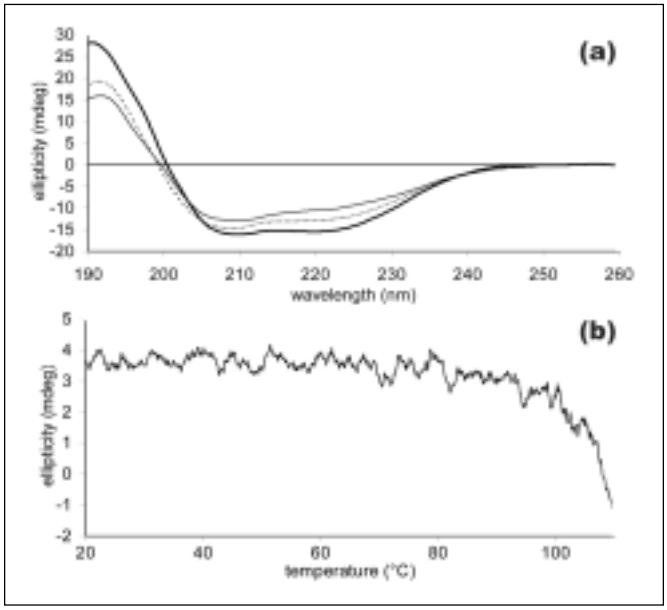

Figure 5—(a) Far-UV circular dichroism (CD) spectra of sunflower albumins (SFAs) (pH 7.0; *I* = 30 m*M*) recorded at 20 °C (thick solid line), 110 °C (thin solid line), and 20 °C after heating up to 110 °C (dashed line); and (b) Far-UV CD temperature scan of SFAs at pH 7.0 (*I* = 30 m*M*), recorded at 200 nm.

Conclusions

Extending the work of Pandya and others (1999) for a single sunflower albumin (SFA8), we have found that the native structure of all SFA proteins is very stable (against pH modification and heat treatment) and their solubility is hardly affected by pH. Generally, the pH of food products ranges from pH 3.0 to 7.0, and the ionic strength varies from 0.02 to 0.2 M (Lakemond and others 2000). This makes SFAs a very attractive group of proteins because minor structure modifications are expected to occur during processing. Sunflower isolates are more likely to occur as a food protein ingredient than SFAs or helianthinin, their major protein components. Knowledge about the physicochemical properties of SFAs or helianthinin allows us to predict and explain the behavior of sunflower protein isolate from the contribution of the individual protein fractions under various conditions. For instance, in foams and emulsions, the soluble proteins govern the functionality of a protein preparation. At low pH, therefore, the functional properties of sunflower isolate are expected to be dominated by the properties of SFAs.

Acknowledgments

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