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Conformational States of Sunflower (*Helianthus annuus*) Helianthinin: Effect of Heat and pH

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The structure and solubility of helianthinin, the most abundant protein of sunflower seeds, was investigated as a function of pH and temperature. Dissociation of the 11S form (hexamer) into the 7S form (trimer) gradually increased with increasing pH from 5.8 to 9.0. High ionic strength ($I = 250$ mM) stabilizes the 11S form at pH > 7.0. Heating and low pH resulted in dissociation into the monomeric constituents (2-3S). Next, the 7S and 11S forms of helianthinin were isolated and shown to differ in their secondary and tertiary structure, and to have denaturation temperatures (T_d) of 65 and 90 °C, respectively. Furthermore, the existence of two populations of the monomeric form of helianthinin with denaturation temperatures of 65 and 90 °C was described. This leads to the hypothesis that helianthinin can adopt two different conformational states: one with $T_d = 65$ °C and a second with $T_d = 90$ °C.

KEYWORDS: ***Helianthus annuus*; sunflower; helianthinin; stability; conformational states; denaturation**

INTRODUCTION

The high protein content (20%) makes sunflower seed an attractive food protein source (*1*). Sunflower seed contains two major groups of proteins, 11S globulin, also known as helianthinin, and 2S albumins, also known as sunflower albumins (SFAs). The two groups are present in a ratio of about 2:1 (11S:2S, respectively) (*2*).

Helianthinin has been reported to be present as a globular oligomeric protein with a molecular weight (MW) of 300−350 kDa (*3*, *4*). Studies on the quaternary structure of helianthinin by electron microscopy and small-angle X-ray scattering indicate that the molecule consists of an arrangement of six spherical subunits into a trigonal antiprism with a maximum dimension of 11 nm (*5*, *6*). As in other 11S seed globulins (pea, faba, soy, or lupin), each subunit consists of an acidic (32−44 kDa) and a basic (21−27 kDa) polypeptide, linked by a single disulfide bond and derived by posttranslational cleavage of a parental protein (*7*−*9*). Besides heterogeneity due to multiple polypeptide chains within a single genotype (*7*, *10*), there are also differences in helianthinin between varieties (*8*, *11*). One sequence of a helianthinin gene (Helianthinin G3 or HAG3) is available. This sequence reveals that helianthinin consists of an acidic chain of 285 amino acids (MW: 32 643 Da) and a basic chain of 188 amino acids (MW: 20 981 Da) linked by a disulfide bond (103−312) (*9*).

Association and dissociation phenomena are a common feature of many 11S seed globulins (*12*, *13*). For instance, 11S globulins from soy bean (*14*), sesame (*15*), kidney bean (*16*), or pea (*17*) have been shown to undergo reversible or irreversible pH-dependent dissociation. Helianthinin association−dissociation has not received much attention. Although dissociation of sunflower 11S into 7S and 2-3S has been reported (*4*, *18*), limited data on the effects of pH and ionic strength on the structure of helianthinin have been published. This research presents a detailed study of the influence of pH and temperature on the dissociation−association behavior of helianthinin and the relation to its solubility. Moreover, the various forms of helianthinin (11S, 7S, and 3S) have been isolated, and their differences at the secondary and tertiary level have been established.

MATERIALS AND METHODS

Protein Isolation. *Defatting and Dephenolizing.* 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) aqueous methanol as described previously (*19*). This procedure yields the defatted dephenolized meal.

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Helianthinin Isolation. The defatted dephenolized meal 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. Next, continuous centrifugation was carried out in a vertical centrifuge type V30-O/703 (Heine; GFT Trenntechnik, Viersen, Germany) at the maximum speed of 3500 rpm. Filter cloths (mesh size 1 μm) were purchased from Lampe Technical Textiles BV in Sneek (Netherlands). Insoluble protein was recovered and washed once [2% (w/v)] at pH 5. Afterward, the pellet was resuspended in water [2% (w/ v)] and stirred for 2 h while keeping the pH at 8.5 by addition of small volumes of 1 N NaOH. Soluble protein was recovered by filter centrifugation (1 μm, 20 °C). The remaining pellet was re-extracted under similar conditions, and the two supernatants were combined. Subsequently, the combined supernatant was diafiltrated using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cutoff of 100 kDa (A/G Technology Corp., Needham, MA) until the conductivity of the retentate remained constant. The retentate was freeze-dried and denoted helianthinin extract. Further purification was performed by gel permeation chromatography. The helianthinin extract was dissolved [1% (w/v)] in 30 mM sodium phosphate buffer (pH 8.0), and 150 mL of the solution was applied, after filtration (0.45 μm), on a Superdex 200 column (68 $\times$ 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^{-1}. The absorbance of the eluate was monitored at 280 nm. Fractions eluting between 1500 and 2500 mL were pooled, diafiltrated as described above, and freeze-dried to yield pure helianthinin. Fractions eluting between 2600 and 3100 mL were also collected and processed in the same way as helianthinin, and were denoted Hel26−31.

Purification of 7S and 11S Forms of Helianthinin. To obtain the 7S and 11S forms of helianthinin, the helianthinin extract was fractionated by gel permeation on a semipreparative Superdex 200 column 16/60 (60 $\times$ 1.6 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with 30 mM sodium phosphate buffer (pH 8.0) at a flow rate of 1 mL min^{-1}. Two peaks were detected at 280 nm. Fractions making up these peaks were individually collected and concentrated to 1.0 mg mL^{-1} with Microcon centrifugal filters YM-10 (Millipore, Etten-Leur, The Netherlands).

Determination of Protein Solubility. The purified helianthinin was dispersed to a final concentration of 4.0 mg mL^{-1} in water, and the pH was adjusted to 8.5 by addition of small volumes of (0.1−1 M) NaOH solutions. The ionic strength was adjusted to 0.03 or 0.25 by adding NaCl. The pH of the helianthinin solution was lowered by adding various volumes of (0.1−1 M) 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 15 800*g* at 20 °C. The protein concentration of the supernatants was determined in triplicate using the Bradford assay (*20*) with bovine serum albumin (BSA) as a standard. Solubility was expressed as proportion (%) of the amount of protein dissolved at pH 8.5. All solubility experiments were performed at least in duplicate.

Protein concentrations as estimated using the Bradford assay (*20*) and the Dumas method were compared and found to be similar (less than 10% difference). For the latter method, a NA 2100 nitrogen analyzer was used according to the instructions of the manufacturer.

Analysis. *Protein Content.* Protein content (N $\times$ 5.3) of the protein preparations was determined by the Kjeldahl method, AACC 46-12 (*21*).

Protein Size and Composition. Protein size and composition were estimated by analytical gel permeation chromatography and gel electrophoresis.

Gel Permeation Chromatography (GPC). Gel permeation chromatography was performed using an Äkta Explorer System (Amersham, Pharmacia Biotech, Uppsala, Sweden). The helianthinin preparations (purified helianthinin, Hel26−31, and the 7S and 11S isolated forms of helianthinin) (0.2−2 mg mL^{-1}) were dissolved at room temperature in 50 mM 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 filter) on a Superdex 200 HR 10/30 (30 $\times$ 1 cm) column and eluted with the same buffer at a flow rate of 0.5 mL min^{-1} at room temperature.

The quaternary structure of helianthinin was also monitored by gel permeation chromatography. The effect of pH on the 7S/11S ratio was studied at two values of ionic strength. In this case, purified helianthinin was dissolved (0.2 mg mL^{-1}) in a NaCl solution (I = 30 and 250 mM) while keeping the pH at 9.0 by addition of small amounts of NaOH solutions (0.1−2 M). The pH of parts of the solution was lowered by adding different amounts of HCl solutions (0.1−2 M) to obtain final pH values of 8.0, 7.0, 6.2, and 5.8. After filtration (0.2 μm filter), the samples (0.2 mL) were applied directly to a Superdex 200 HR 10/30 column and eluted at a flow rate of 0.5 mL min^{-1} with the following buffers matching the pH of the samples: 30 mM sodium acetate buffer, pH 5.8; 30 mM sodium phosphate buffer, pH 6.2; 30 mM sodium phosphate buffer, pH 7.0; 30 mM sodium phosphate buffer, pH 8.0; 30 mM sodium borate buffer, pH 9.0. The ionic strength of the buffers was adjusted to 30 and 250 mM by adding NaCl.

The effect of ionic strength on the 7S/11S ratio was studied at pH 7.0. Purified helianthinin was dissolved (0.2 mg mL^{-1}) in 30 mM sodium phosphate buffer of which the ionic strength was varied by adding NaCl (I = 50, 250, 500, 1000, and 1250 mM). After filtration (0.2 μm filter), the samples (0.2 mL) were applied to a Superdex 200 HR 10/30 column and eluted at a flow rate of 0.5 mL min^{-1} with buffers matching the ionic strength of the samples.

The column was calibrated using protein markers ranging from 13 to 2000 kDa (Amersham, Pharmacia Biotech, Uppsala, Sweden): ribonuclease A (13 700 Da), ovalbumin (43 000 Da), BSA (67 000 Da), aldolase (158 000 Da), catalase (232 000 Da), ferritin (440 000 Da), and blue dextran (2 000 000 Da). The absorbance of the eluate was monitored at 214 and 280 nm.

Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Mini-PROTEAN II electrophoresis system (BIO-RAD Laboratories), following the instructions of the manufacturer. Protein samples of 10−15 μg were dissolved in either reducing or nonreducing sample buffer, and were applied to homogeneous 12% gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue. For calibration, molecular weight markers were used ranging from 14 to 94 kDa (Amersham, Pharmacia Biotech, Uppsala, Sweden): α-lactalbumin (14 400 Da), soybean trypsin inhibitor (20 100 Da), carbonic anhydrase (30 000 Da), ovalbumin (43 000 Da), BSA (67 000 Da), and phosphorylase b (94 000 Da). SDS-PAGE was also performed according to the method of Schägger and Jagow (*22*) to analyze low molecular weight proteins. Protein samples of 10−15 μg were applied to precast 16.5% Tris-tricine gels (Bio-Rad laboratories). Markers ranging from 3.5 to 26.6 kDa were applied in this case (Bio-Rad laboratories): bovine insulin (3500 Da), aprotinin (6500 Da), lysozyme (14 400 Da), myoglobin (16 950 Da), and triosephosphate isomerase (26 600 Da).

Isoelectric Focusing (IEF). Isoelectric focusing was performed on a LKB 2117 MULTIPHOR II isoelectric focusing module (Pharmacia LKB Biotechnology), following the instructions of the manufacturer. Purified helianthinin (10−15 μg) was dissolved in sample buffer, and was applied to IEF 3.0−9.0 gels (Servalyt Precotes 150 μm, 125 $\times$ 125 mm, Serva, Heidelberg, Germany). The gels were run for 3 h following the instruction of the manufacturer. IEF standards (Amersham, Pharmacia Biotech, Uppsala, Sweden) were used to estimate the isoelectric pH (pI) of each band after staining with Coomassie Blue G250: trypsinogen (pI: 9.30), lentil lectin-basic band (pI: 8.65), lentil lectin-middle band (pI: 8.45), lentil lectin-acidic band (pI: 8.15), myoglobin-basic band (pI: 7.35), myoglobin-acidic band (pI: 6.85), human carbonic anhydrase B (pI: 6.55), bovine carbonic anhydrase B (pI: 5.85), β-lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI: 4.55), and amyloglucosidase (pI 3.50).

Differential Scanning Calorimetry (DSC). Calorimetric studies were performed using a VP-DSC MicroCalorimeter (MicroCal Inc., Northhampton, MA). Thermograms were recorded from 20 to 130 °C with a heating rate of 1 °C min^{-1}. Experiments were performed with helianthinin, at concentrations of 0.5−4.0 mg mL^{-1} at several pH values: pH 3.0 (10 mM sodium phosphate buffer), pH 7.0 (10 mM sodium phosphate buffer), and pH 8.5 (10 mM sodium borate buffer). The final ionic strengths (10, 30, or 250 mM) of the buffers were adjusted by adding NaCl. The protein concentration of the solutions

was estimated by absorbance measurement at 280 nm, using sunflower isolate (*19*) as standard. All measurements were carried out in duplicate.

Circular Dichroism (CD) Spectroscopy. The protein concentration of the solutions was routinely estimated by absorbance measurement at 280 nm, as described above.

Far-UV CD. Far-UV CD spectra of helianthinin samples were recorded at 20, 110, and 20 °C after heat treatment at 110 °C, as averages of 10 spectra on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at several pH values: pH 3.0 (10 mM sodium phosphate buffer), pH 7.0 (10 mM sodium phosphate buffer), and pH 8.5 (10 mM sodium borate buffer). The final ionic strengths (30, 250 mM) of the buffers were adjusted by adding NaF. Quartz cells with an optical path length of 1 mm and 0.2 mm at protein concentrations of approximately 0.04 and 0.1 mg mL^{-1}, respectively, were used. The scan interval was 180−260 nm, the scan speed was 100 nm min^{-1}, the data interval was 0.2 nm, the bandwidth was 1.0 nm, the sensitivity was 20 mdeg, 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 estimate the secondary structure content of the protein using a nonlinear regression procedure as described in detail by Pots and co-workers (*23*).

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

Near-UV CD. Near-UV CD spectra of 2.0−3.0 mg of protein mL^{-1} solutions of helianthinin were recorded at 20 °C as averages of 25 spectra on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at pH 3.0 (10 mM sodium phosphate buffer) and pH 7.0 (10 mM sodium phosphate buffer). The final ionic strengths (30 or 250 mM) of the buffers were adjusted by adding NaF. Quartz cells with an optical path length of 10 mm were used. The scan speed was 50 nm min^{-1}, the scan interval was 250−350 nm, the data interval was 0.5 nm, the bandwidth was 1.0 nm, the sensitivity was 10 mdeg, and the response time was 0.25 s. Near-UV CD spectra of purified helianthinin were also recorded at 105 and 20 °C (after heating at 105 °C during 10 min). Spectra were corrected by subtracting the spectrum of a protein-free sample obtained under identical conditions.

Changes in the thermal stability of the tertiary structure of sunflower proteins were also monitored by measuring the ellipticity at 285 nm as a function of temperature at a heating rate of 1 °C min^{-1}.

Amino Acid Analysis. Amino acid analysis was performed after protein hydrolysis using an amino acid analyzer equipped with a ninhydrin detection system. Acid hydrolysis was carried out with 6 M HCl during 22 h at 105−110 °C. To analyze cysteine and methionine, the sample underwent oxidation with performic acid during 16 h at 0−5 °C, followed by acid hydrolysis with 6 M HCl during 22 h at 105−110 °C. For tryptophan determination, alkaline hydrolysis was performed with 4.2 M NaOH during 22 h at 105−110 °C.

RESULTS

Protein Composition. The protein content of the helianthinin extract, obtained as described under Materials and Methods, was above 85%. The helianthinin extract was subjected to gel permeation chromatography, both on an analytical as well as on a preparative scale. Analytical gel permeation chromatography (**Figure 1**) showed four peaks with elution volumes of approximately 10.3 ± 0.2, 11.4 ± 0.2, 14.0 ± 0.3, and 17.0 ± 0.2 mL. Calibration of the column revealed apparent molecular weights of 300, 150, 45, and 14 kDa, respectively. The results obtained with preparative chromatography closely resembled those obtained using analytical chromatography, also showing four peaks. Fractions (1500−2500 mL) corresponding to the peaks at 10.3 and 11.4 mL were pooled and denoted as purified helianthinin.

Analytical GPC of the purified helianthinin preparation showed only the two peaks at 10.3 ± 0.2 and 11.4 ± 0.2 mL, denoted 11S and 7S in **Figure 1**. SDS-PAGE of these fractions

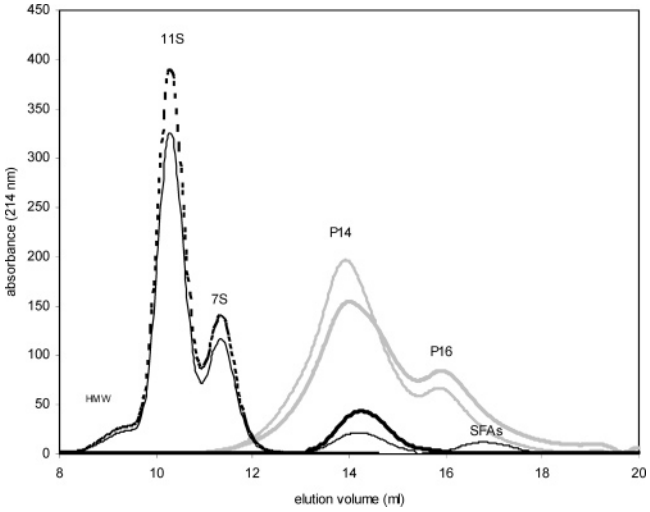

Figure 1. Chromatograms of helianthinin preparations under various conditions. Analysis at pH 6.9 (50 mM sodium phosphate buffer). Helianthinin extract (thin black line), purified helianthinin (dashed black line), purified helianthinin after heat treatment (100 °C, 10 min; gray line), helianthinin after acid treatment (pH 3.0, 1 h; thick gray line), and Hel26−31 preparation (thick black line).

confirmed the identity of helianthinin with the presence of bands as those previously described (*7*, *10*, *19*) (**Figure 2A**). IEF of these fractions (10.3 ± 0.2 and 11.4 ± 0.2 mL) displayed several bands with pI's between 5.0 and 5.9 (**Figure 2B**).

The fraction that eluted between 2600 and 3100 mL on the preparative column (Hel26−31) corresponded to the peak eluting at 14 ± 0.3 mL on the analytical column (**Figure 1**) and had an estimated MW of 45 kDa and was denoted as P14. SDS-PAGE of this fraction under reducing conditions showed bands with approximate molecular weights of 24, 30, and 40 kDa as described for helianthinin (*19*) (results not shown). The 30 kDa band was the main band.

Tricine SDS-PAGE of the peak eluting at 17 mL showed two bands with an estimated MW of approximately 12 and 15 kDa (**Figure 2C**). Proteins with these molecular weights have been reported to be sunflower albumins (SFAs) (*24*, *25*).

Protein Solubility. Protein solubility was measured in the pH range 2.0−8.5, at $I = 30$ and 250 mM. The solubility of purified helianthinin as a function of pH is shown in **Figure 3A**. At low ionic strength ($I = 30$ mM), helianthinin exhibits a bell-shaped curve with a minimum solubility at pH 4.0−5.5. At high ionic strength ($I = 250$ mM), helianthinin is almost insoluble at pH < 5.0. The solubility of helianthinin at pH 3.0 was found to be strongly affected by ionic strength (**Figure 3B**). The solubility remained more or less constant between 0 and 150 mM and exhibited a decrease above 150 mM.

Structural Changes at Various pH Values. *Secondary Folding.* Far-UV CD spectra of purified helianthinin were recorded at pH 7.0 ($I = 30$ and 250 mM), pH 8.5 ($I = 10$ mM), and pH 3.0 ($I = 30$ mM) at 20 °C (**Figure 4**). The characteristic features of the spectra at neutral and weakly alkaline pH values are a minimum at about 210 nm and a zero crossing around 200 nm. On the basis of comparison with reference spectra (*26*), helianthinin seems to mainly consist of α-helical structures. Using curve-fitting procedures, the secondary structure content was estimated, confirming the high content of α-helical structures. At both ionic strengths (30 and 250 mM) at pH 7.0, α-helical parts account for 60%, and nonstructured parts for approximately 10% of the secondary structure of helianthinin. No β-sheet elements were present. Far-UV CD spectra of

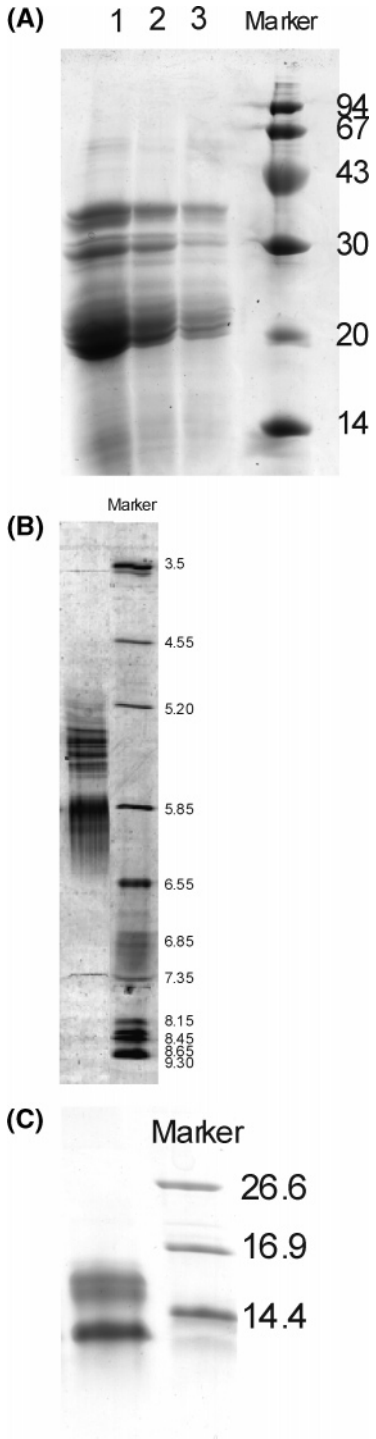

Figure 2. (**A**) SDS-PAGE patterns of helianthinin under reduction conditions. Lane 1, purified helianthinin; lane 2, 11S form of helianthinin; lane 3, 7S form of helianthinin. (**B**) Isoelectric focusing of purified helianthinin. Only the relevant part of the gel is shown. (**C**) Tricine SDS-PAGE of SFAs in **Figure 1**. Only the relevant part of the gel is shown.

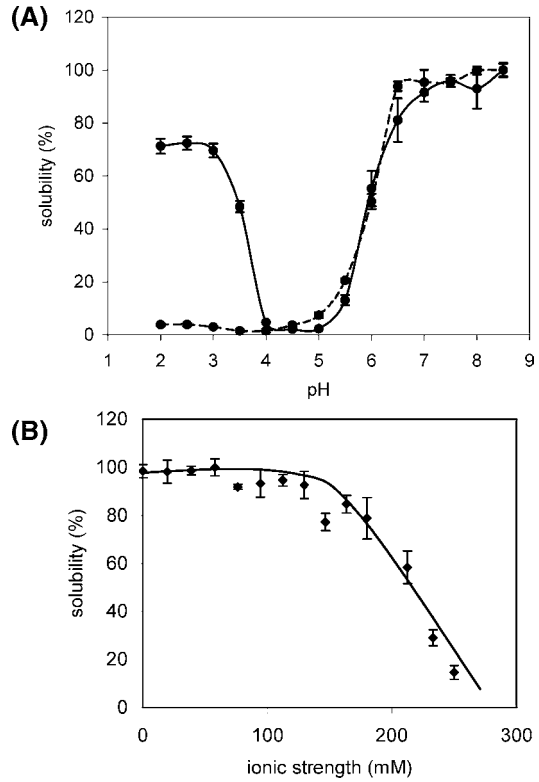

Figure 3. (**A**) pH-dependent solubility profiles of purified helianthinin. I = 30 mM (solid line) and 250 mM (dashed line). Expressed as proportion (%) of the amount of protein dissolved at pH 8.5. (**B**) Purified helianthinin solubility versus ionic strength at pH 3.0. Expressed as proportion (%) of the amount of protein dissolved at pH 3.0 (I = 30 mM).

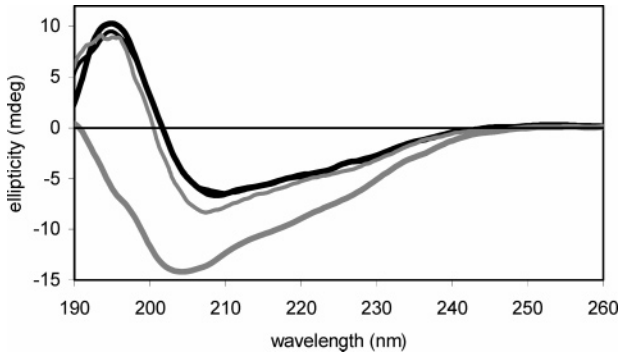

Figure 4. Far-UV CD spectra of purified helianthinin. The experiments were performed at pH 7.0 (I = 30 mM; black thin line), pH 7.0 (I = 250 mM; black thick line), pH 8.5 (I = 10 mM; thin gray line), and pH 3.0 (I = 30 mM; thick gray line).

helianthinin at pH 8.5 did not differ much from those at pH 7.0; the estimated amount of nonstructured protein was about 5−10% lower at pH 7.0. However, at pH 3.0, the far-UV CD spectrum is totally altered. The zero crossing has shifted from 200 to 190 nm, the spectrum shows only negative ellipticity, and an estimation of the secondary structure content revealed the presence of approximately 50% nonstructured protein.

Tertiary Folding. At neutral pH, the near-UV CD spectra of purified helianthinin (**Figure 5**) at both ionic strengths (30 and 250 mM) were very similar. They showed a maximum at 285 nm and a shoulder at 292 nm, which are both probable due to mainly tryptophan and some tyrosine contributions (*27*, *28*). The intensity of the spectra was slightly lower at I = 30 mM as compared to I = 250 mM, which generally points at a minor destabilization of the protein structure (*29*).

The near-UV CD spectrum of helianthinin at pH 3.0 is clearly different from that at pH 7.0. As compared to pH 7.0, a drastic decline of intensity over the full range is observed pointing to a total unfolding of the tertiary structure.

Quaternary Structure. As mentioned before, gel permeation chromatography (GPC) showed two peaks for purified helianthinin. The MW of these two peaks suggests partial dissociation of the 11S form into a 7S form, as previously reported (*4*, *30*). To gain a better insight into this dissociation behavior, it was

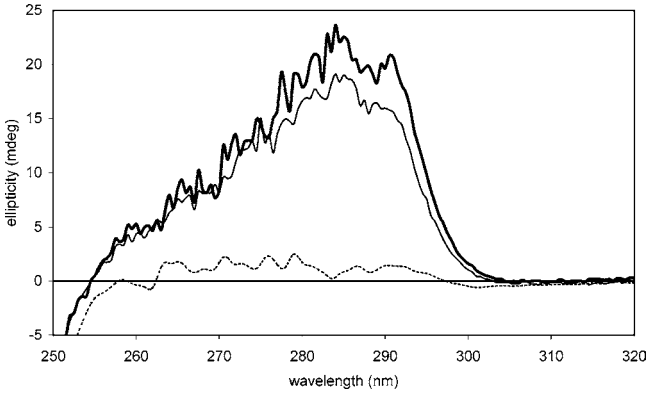

Figure 5. Near-UV CD spectra of purified helianthinin. The experiments were carried out at pH 7.0 ($I = 30$ mM; thin line), pH 7.0 ($I = 250$ mM; thick line), and pH 3.0 ($I = 30$ mM; dotted line) at 20 °C.

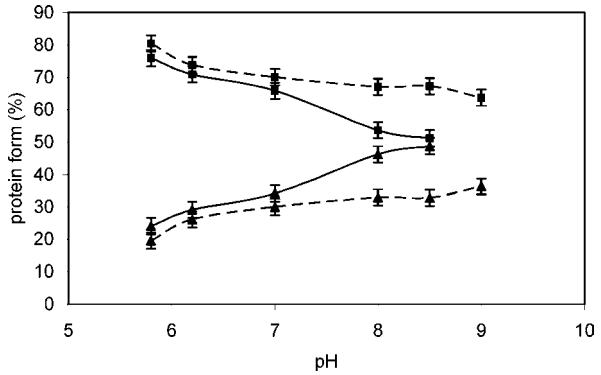

Figure 6. Proportion of 11S (■) and 7S (▲) forms of helianthinin as a function of pH. At $I = 30$ mM (solid line) and $I = 250$ mM (dashed line).

studied how the ratio 11S/7S is affected by pH (5.8 to 9.0; $I =$ 30 and 250 mM) and by ionic strength (50 to 1250 mM; pH 7.0) using GPC.

Figure 6 shows that for $I = 30$ mM the proportion of 11S decreases with increasing pH. At pH 9.0, an additional peak, eluting at 8.7 ± 0.4 mL, was observed in the gel permeation chromatogram (no further data shown). This peak is likely due to aggregation of helianthinin into a higher molecular weight form, presumably 15−18S (*30*−*33*) and is, therefore, not illustrated in **Figure 6**. Small amounts (1−5%) of this aggregate were also found at other pH values. The amount of 11S also decreased with increasing pH at a higher ionic strength ($I =$ 250 mM), although to a lesser extent. However, no aggregation into 15S was found above pH 8.0. No effect of the ionic strength on the 11S/7S ratio was observed at pH 7.0, even up to values of 1250 mM (results not shown).

To further investigate differences in quaternary structure, the 7S and 11S forms of helianthinin were isolated by preparative GPC and further analyzed. SDS-PAGE did not show differences between the subunits of the 11S and 7S forms (**Figure 2A**). The near and far-UV CD spectra of these forms of helianthinin were apparently similar to those of the nonfractionated helianthinin. However, the intensities of the near-UV CD spectra of 7S were lower than those of 11S, and the far-UV CD spectra revealed a higher content of nonstructured protein (i.e., at pH 8.0 and $I = 30$ mM, 25% versus 3%) in the 7S form as compared to the 11S form (spectra not shown).

The effect of time on helianthinin dissociation was also studied. The 11S form of helianthinin was monitored (pH 7; $I =$ 30 mM) by GPC for 1 week. It was found that at the conditions applied, dissociation is a very slow process because

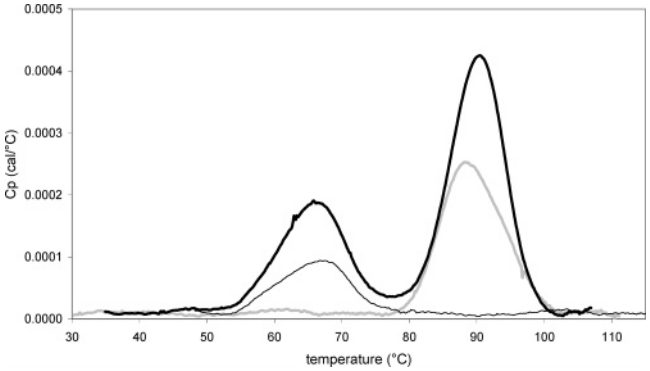

Figure 7. DSC thermograms of helianthinin. Purified helianthinin (thick line), purified 11S form (gray line), and 7S form (thin line) of helianthinin at pH 8.5 and $I = 10$ mM. Data have not been normalized.

more than 5 days were needed to dissociate 50% of the 11S form into the 7S form. The isolated 7S form of helianthinin was also monitored as a function of time, and no reassociation into 11S was observed, pointing to an essentially irreversible dissociation of 11S (results not shown).

GPC of the soluble part of helianthinin at pH 3.0, as obtained in the solubility experiment (**Figure 3A**), indicated that helianthinin is fully dissociated into two kinds of smaller fragments: P14 and a smaller fragment eluting at 16 mL (denoted P16; estimated MW 25 kDa).

Structural Changes as a Function of Temperature. At pH 7.0, severe heat-induced aggregation of helianthinin occurred, and therefore other conditions were tested. In many cases, aggregation can be avoided by keeping the ionic strength of the solvent low and by using pH values far from the isoelectric pH (*34*). Therefore, DSC scans were performed at pH 8.5 ($I =$ 10 mM). **Figure 7** shows the DSC thermograms of the purified helianthinin and its 7S and 11S forms. Helianthinin showed two endothermic transitions at approximately 65 and 90 °C. All of the transitions were irreversible as observed from rescanning of the samples (not shown). The denaturation temperatures were independent of protein concentration (0.5−4.0 mg mL^{-1}) and of scan rate employed (0.5−1.5 °C min^{-1}). At pH 3.0, helianthinin seemed already completely denatured as deduced from the absence of endothermic transitions (results not shown).

To investigate the nature of the two endothermic transitions observed, purified helianthinin was heated ($I = 10$ mM; pH 8.5) up to 65 °C during 5, 30, and 60 min and subsequently rescanned. **Figure 8A** shows that upon increasing the preheating time at 65 °C, the area of the first DSC peak of helianthinin decreased. The second peak is not affected by preheating at 65 °C. Subsequent GPC analyses of the samples showed a progressive disappearance of the 7S form by heating at 65 °C, whereas the area of 11S peak remained constant (**Figure 8B**). The disappearance of the peak was found to be proportional to the time that helianthinin was heated at 65 °C. Furthermore, isolated 7S and 11S forms showed denaturation temperatures of 65 and 90 °C, respectively (**Figure 7**). These experiments demonstrate that the 7S form of helianthinin unfolds at a lower temperature than the 11S form. In **Figure 8B**, it can be also observed that heating of purified helianthinin up to 65 °C resulted in protein dissociation into P14, suggesting that this peak corresponds to an even smaller dissociated part of helianthinin.

To further monitor changes in the secondary structure of helianthinin as a function of temperature, far-UV CD temperature scans were recorded at 200 nm from 20 to 110 °C (**Figure 9A**). The ellipticity was monitored at this wavelength because

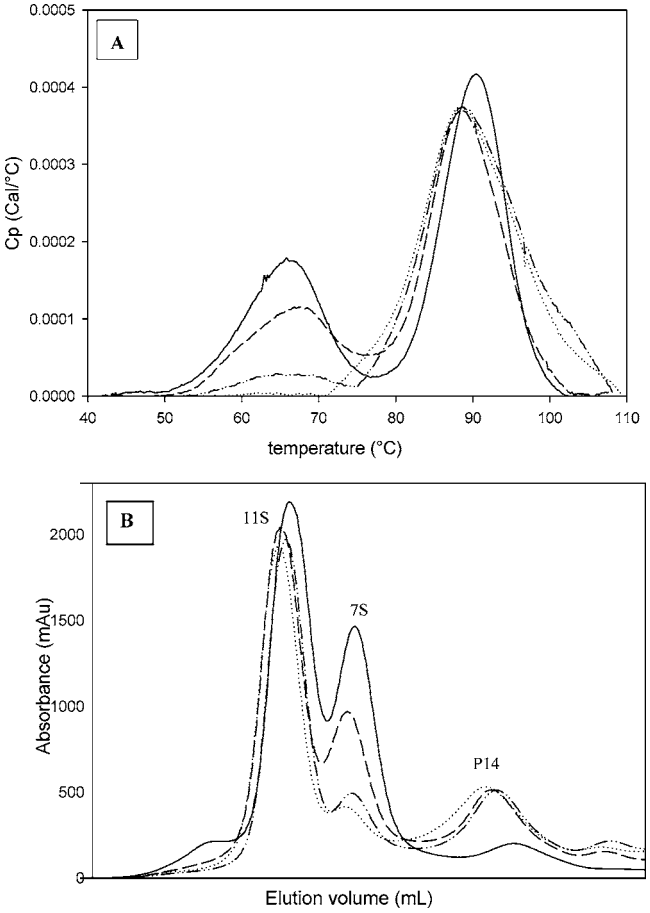

Figure 8. (**A**) DSC thermograms of purified helianthianin after preheating at 65 °C. At pH 8.5 ($I = 30$ mM) without previous heating (solid line) and after heating at 65 °C for 5 min (dash line), 30 min (dotted-dashed line), and 60 min (dotted line). (**B**) GPC chromatograms of purified helianthinin after heating at 65 °C. At pH 8.5 ($I = 30$ mM) without previous heating (solid line) and after heating at 65 °C for 5 min (dash line), 30 min (dotted-dashed line), and 60 min (dotted line).

it showed the largest changes as a function of temperature (**Figure 9B**). **Figure 9A** indicates that at pH 8.5 two successive transitions occurred with midpoints at approximately 65 and 90 °C, respectively. These data are very similar to the DSC results. Isolated 11S and 7S forms of helianthinin showed a single transition at approximately 90 and 65 °C, respectively (**Figure 9A**), also in accordance with the DSC results.

At pH 3.0, helianthinin showed only a slight change in ellipticity upon heating. Using curve-fitting procedures, a content of nonstructured protein was found for heat-treated (110 °C) helianthinin at pH 7.0 (60%) similar to that for unheated helianthinin at pH 3.0 (50%).

The near-UV CD spectra of helianthinin at pH 7.0 ($I = 30$ mM) after heating to 105 °C also resembled the spectrum of helianthinin at pH 3.0 ($I = 30$ mM) (**Figure 5**), although the decline of intensity over the full range was somewhat more drastic after heating (no further data shown).

DISCUSSION

Structure of Helianthinin. Subunit Arrangement at Various Conditions. Like other 11S seed globulins, helianthinin seems to dissociate into subunits according to the following scheme:

$$11S \rightleftharpoons 7S \rightleftharpoons 3\text{-}2S$$

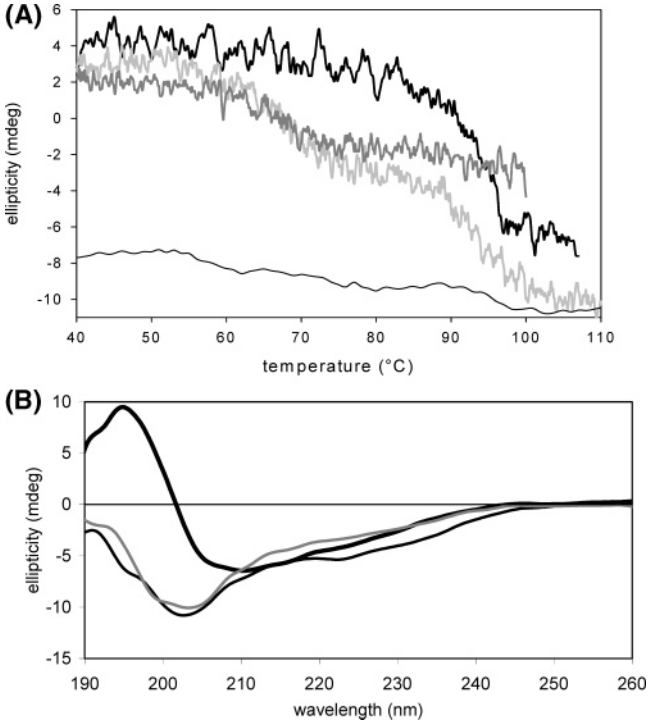

Figure 9. (**A**) Far-UV CD temperature traces of purified helianthinin at 200 nm at various conditions. At pH 3.0 ($I = 30$ mM; thin solid line); pH 8.5 ($I = 10$ mM; light gray line); isolated 7S form at pH 8.5 ($I = 10$ mM; dark gray line); isolated 11S form at pH 8.5 ($I = 10$ mM; thick solid line). (**B**) Far-UV CD spectra of purified helianthinin at several temperatures. Spectra were recorded at 20 °C (thick solid line), 110 °C (thin solid line), and 20 °C after heating at 110 °C (gray line) (pH 7.0, $I = 30$ mM).

Dissociation of helianthinin at acidic conditions has been reported (*18*, *35*, *36*), but the dissociation products were not identified and no data on changes in the alkaline, neutral, and mild acid pH range were reported.

Our results show that the quaternary structure of helianthinin is modulated by both ionic strength and pH. Dissociation of the 11S form into the 7S form gradually increased with increasing pH from 5.8 to 9.0 at low (30 mM) and high (250 mM) ionic strength. In contrast to other publications (*36*, *37*), which have reported that in solutions of low ionic strength ($I < 300$ mM) the 11S form of heliathinin dissociates into the 7S form, this study shows that both forms of helianthinin are present at pH 7.0 in a broad ionic strength range (30−1250 mM). However, high ionic strengths seem to stabilize the 11S form of helianthinin at pH values above 7.0, probably by decreasing electrostatic repulsion.

Next to pH and ionic strength, also time seems to be an important parameter. We found 11S dissociates into 7S in time at fixed conditions. Although no association of 7S into 11S was observed, the extremely low rate of the process leaves open the possibility of an equilibrium, instead of an irreversible process. Schwenke et al. (*4*) suggested an 11S/7S equilibrium associated with a partially irreversible dissociation of the 11S form into the 7S form of helianthinin. It may therefore be that, from a thermodynamic point of view, the dissociation of 11S into 7S under nondenaturing conditions is essentially a reversible process.

Both low pH and heating (**Figure 1**) induced dissociation of helianthinin into two other protein fragments, P14 and P16. P14 has an estimated MW of 45 kDa. Because the monomeric subunit of helianthinin has a MW of about 50 kDa (300 kDa/6), it can be assumed that this fragment corresponds to the

monomeric subunit. The amino acid composition of P14 (Hel26−31) was found to be identical to that of helianthinin (results not shown). Furthermore, SDS-PAGE under reducing conditions showed the same MW distribution as helianthinin (results not shown).

Assuming the generally adopted 6(AB) oligomeric structure for the 11S form, in which A and B are the acidic and basic polypeptides, respectively, these results show that the dissociated helianthinin fragments observed may correspond to the trimer 3(AB) for the 7S form and to the monomer (AB) for the 3S form. The identity of the P16 fragment remains unclear.

The dissociation of helianthinin involves significant changes in secondary and tertiary structure. The lower intensity of the near-UV CD spectrum for the 7S form points to destabilization of tertiary structure. Furthermore, dissociation into 7S seems to be associated with a higher amount of nonstructured secondary folding as estimated from far-UV CD. It could also be observed that a somewhat higher amount of nonstructured protein is present in helianthinin at pH 8.5 as compared to that at pH 7.0, which is in agreement with the higher ratio 7S/11S at alkaline pH values (**Figure 6**). Far-UV CD spectra of the monomer (3S; Hel26−31) at pH 8.5 revealed that the content of nonstructured protein was close to that found for helianthinin at pH 3.0. These results indicate that dissociation of helianthinin is either the cause or the result of conformational changes at both secondary and tertiary levels.

Heat Denaturation of Helianthinin. The DSC-profiles of helianthinin at pH 8.5 (I = 10 mM) showed two peaks at temperatures of about 65 and 90 °C, for the trimeric and hexameric forms of helianthinin, respectively. If dissociation of the hexamer or the trimer occurs during thermal denaturation, the denaturation temperature (T_d) should rise with increasing protein concentration (*34*, *38*, *39*). Variation of the protein concentration (0.5−3.0 mg mL^{-1}) did, however, not result in significant changes in the values of T_d. If the reacting species is known to be oligomeric at ambient temperature and T_d is concentration independent, it may be concluded that either the oligomer has become monomeric by the time the denaturation temperature is reached or that no dissociation or association accompanies heat denaturation (*39*). Gel permeation chromatography of samples submitted to thermal denaturation indicated that irreversible dissociation had occurred for both the 7S and the 11S forms. Therefore, our results point toward the occurrence of dissociation before denaturation.

Formally, the application of thermodynamic equations is only allowed for reversible two-state transitions. These conditions are not entirely fulfilled here because the thermal denaturation is not fully reversible. Nevertheless, many empirical results provide some measure of validity of the application of equilibrium thermodynamics to apparently irreversible processes (*39*). Furthermore, Schwenke et al. (*40*) have demonstrated that the thermal unfolding of 11S globulin from soy, faba, sunflower, and rapeseeds can be described adequately by a two-state model. We also observed T_d to be independent of the scan rate, indicating chemical equilibrium during thermal denaturation. Thermodynamic data were also obtained from the far-UV CD thermal unfolding curves of helianthinin according to the model of Van Mierlo et al. (*41*) and from DSC data by applying equilibrium thermodynamic expressions (*39*, *42*, *43*). **Table 1** shows the values of the van't Hoff enthalpy (ΔH_{vH}) and T_d obtained from the CD-unfolding curve and DSC together with the calorimetric enthalpy values (ΔH_{cal}). An average molar mass of the cooperative unit of 25 400 g mol^{-1} was taken as suggested by Schwenke et al. (*40*). As can be calculated from **Table 1**, the ratio $(\Delta H_{cal})/(\Delta H_{vH})$ obtained for the 7S and 11S forms of helianthinin is close to unity, which is an indication that the denaturation can be described with a two-state model.

Table 1. Transition Temperatures and Enthalpies of Denaturation of Helianthinin at pH 8.5 (I = 10 mM) As Measured by DSC and Far-UV CD Temperature Scanning at 200 nm

		DSC			CD	
protein	pH	ΔH_{cal} (kJ mol^{-1})	ΔH_{vH} (kJ mol^{-1})	T_d (°C)	ΔH_{vH} (kJ mol^{-1})	T_d (°C)
11S	8.5	514 ± 53	455 ± 30	90.1 ± 0.7	456 ± 13	90.9 ± 0.9
7S	8.5	347 ± 27	311 ± 29	66.4 ± 1.1	299 ± 6	66.5 ± 0.1
3S $(P14)_{90}$ [a]	8.5	100 ± 14	395 ± 32	89.6 ± 0.2		
3S $(P14)_{65}$ [a]	8.5	67 ± 5	325 ± 22	64.9 ± 0.9		

[a] Calculated independently for the peaks with denaturation temperatures of 65 and 90 °C.

DSC experiments demonstrate that the trimer (7S) denatures at a lower temperature than the hexamer (11S). This behavior was also found for soy glycinin (*44*). Danilenko (*45*) explained the different denaturation temperatures of soy glycinin based on the lower free energy of the 11S form as compared to the 7S form of soy glycinin. The lower amount of tertiary structure of 7S according to the near-UV CD and the higher amount of random coil for 7S helianthinin as estimated by far-UV CD are consistent with the thermodynamic differences found for 7S and 11S. Although the monomer, 3S (Hel26−31), gave much lower ΔH_{cal} values than the oligomeric molecules, it shows denaturation temperatures similar to those of 7S and 11S (**Table 1**), pointing to the existence of two populations of monomers. Furthermore, the ΔH_{vH} values of the 65 and 90 °C populations of monomers did not significantly differ from the ΔH_{vH} values of 7S and 11S. Hence, the same unfolding seems to take place in which less energy is involved. This result would imply that only a small fraction of the monomer exists in the folded form as indicated by the higher content of nonstructured protein found using far-UV CD. Therefore, the determined protein concentration of the monomer for the DSC analysis is likely an overestimation, because both folded and unfolded monomers are taken into account when only the folded monomers contribute to the endothermic transitions. In addition, the ratio $(\Delta H_{cal})_{11S}/(\Delta H_{cal})_{7S}$ was equal to the ratio $(\Delta H_{cal})_{3S(90°C)}/(\Delta H_{cal})_{3S(65°C)}$. These results indicate that the two populations of monomers can presumably be assigned as subunits of the oligomeric molecules (11S and 7S).

Solubility of Helianthinin. The solubility of helianthinin as a function of pH is in agreement with previous publications, which reported a minimum between 4.0 and 5.5 for pure helianthinin and other sunflower protein products containing helianthinin (*46*−*51*). The decreased solubility of helianthinin at pH 3.0 (I = 30 mM) can be attributed to acid-induced denaturation and dissociation of the protein. At high ionic strength (I = 250 mM), helianthinin is almost insoluble below its pI, as can be explained by the decrease in the distance at which electrostatic repulsion acts at high ionic strength, thus allowing the unfolded proteins to approach each other closely enough to form aggregates via mainly hydrophobic interactions. A similar trend has been found by several authors (*46*, *47*, *52*, *53*).

Comparison with previous studies on soy glycinin (*14*, *54*) shows the resemblance between the physicochemical behavior of helianthinin. The stabilizing effect of NaCl, for instance, seems to be a common feature for both proteins. Moreover, the 11S form of these proteins dissociates at acid pH values, and the flat DSC scan reveals the acid denaturation of both proteins

at pH ≤ 3 (*54*). Soy glycinin and helianthinin also share several structural similarities. The far-UV CD spectra of both proteins at neutral pH are also very similar. They exhibit a negative extreme at 208−210 nm and a zero crossing around 200 nm. Furthermore, the secondary folding of both proteins was estimated to mainly consist of α-helical structures (*14*). The near-UV CD spectrum of helianthinin at neutral pH resembles that of soy glycinin (*14*). Both show a positive ellipticity between 260 and 300 nm with a maximum at 285 and a resolved shoulder at 292 nm. Because CD spectra, especially the near-UV, can be seen as a fingerprint of a protein (*27*), this result may indicate a similar tertiary folding of these two seed storage proteins.

The results described in this study lead to the hypothesis that helianthinin can adopt two different conformational states: one state with a denaturation temperature of 65 °C and a second state with a denaturation temperature of 90 °C. Furthermore, the observed slow dissociation of 11S to 7S may also occur with other 11S-type seed proteins, such as those from sesame seed, safflower seed, groundnut, linseed, or poppy seed. However, further research with each individual 11S-type seed protein should be performed to confirm this hypothesis.

ABBREVIATIONS USED

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IEF, isoelectric focusing; GPC, gel permeation chromatography; CD, circular dichroism; DSC, differential scanning calorimetry; ΔH_{cal}, calorimetric enthalpy; ΔH_{vH}, van't Hoff enthalpy; T_d, denaturation temperature; Hel26−31, fraction eluting between 2600 and 3100 mL (preparative GPC); P14, peak eluting at 14 mL (analytical GPC); P16, peak eluting at 16 mL (analytical GPC); S, Svedberg sedimentation coefficient; CGA, chlorogenic acid; SFAs, sunflower albumins; BSA, bovine serum albumin; MW, molecular weight; *I*, ionic strength; pI, isoelectric pH.

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