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Emulsion Properties of Sunflower (*Helianthus annuus*) Proteins

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Emulsions were made with sunflower protein isolate (SI), helianthinin, and sunflower albumins (SFAs). Emulsion formation and stabilization were studied as a function of pH and ionic strength and after heat treatment of the proteins. The emulsions were characterized with respect to average droplet size, surface excess, and the occurrence of coalescence and/or droplet aggregation. Sunflower proteins were shown to form stable emulsions, with the exception of SFAs at neutral and alkaline pH values. Droplet aggregation occurred in emulsions made with SI, helianthinin, and SFAs. Droplet aggregation and subsequent coalescence of emulsions made with SFAs could be prevented at pH 3. Calcium was found to cause droplet aggregation of emulsions made with helianthinin, at neutral and alkaline pH values. Treatments that increase conformational flexibility of the protein molecule improved the emulsion properties of sunflower proteins.

KEYWORDS: Sunflower; *Helianthus annuus*; helianthinin; albumin; protein; emulsion; denaturation

INTRODUCTION

The global demand for protein is increasing, and as a consequence, there is a need for new sources of food proteins. Vegetable proteins are an economic and versatile substitute for animal proteins as a functional ingredient in food formulations. Oilseeds are the most important source of vegetable protein ingredients. Up to now, soy protein is the main oilseed protein used as a functional ingredient in foods such as bakery products, milk substitutes, and meat products. However, sunflower proteins might be a good alternative in view of, among other things, their widespread availability in areas where soy is not or only slightly produced. Furthermore, sunflower seeds have been reported to be low in or devoid of antinutritional factors (ANFs), e.g. protease inhibitors, cyanogens, glusosinolates, etc. (*1*).

The functional properties of sunflower proteins have been studied in the past, which has revealed good emulsification and foaming properties (*2*−*10*), and poor gelling properties (*10*−*13*). Many of the studies dealing with the functionality of sunflower proteins were, however, performed with protein products, the extent of denaturation of which was marginally or not studied. In some cases, however, the isolation procedures must have resulted in severe protein denaturation and subsequent modification of protein functionality (*14*). In addition, some of the protein products investigated contained phenolic compounds, which are known to interact and form complexes with proteins, thereby affecting protein functionality (*15*, *16*). Furthermore, most of the research investigating the emulsion properties has been performed with all of the sunflower protein, i.e., the soluble as well as the insoluble fraction. Although, the insoluble protein is accounted for in the total concentration, its contribution to protein functionality is usually very low (*17*, *18*). Therefore, in this study functionality tests were performed with gently purified proteins and in particular with the soluble fraction.

The two main groups of sunflower proteins are 11S globulin, also known as helianthinin, and 2S albumins, also known as sunflower albumins (SFAs). The currently most accepted model of helianthinin (11S), at neutral pH, consists of an arrangement of six spherical subunits into a trigonal antiprism (*19*). The monomeric subunits consist of an acidic (32−44 kDa) and a basic (21−27 kDa) polypeptide linked by a disulfide bond (*20*, *21*). The structure of helianthinin can be modulated by ionic strength and pH, and helianthinin may occur as a monomer, trimer, or hexamer or in higher aggregated forms (*22*). Sunflower albumins are basic proteins with a molecular mass in the range 10−18 kDa (*23*−*25*). The physicochemical properties of sunflower proteins have been characterized previously (*22*, *26*).

One of the primary functional requirements of many food proteins is the ability to form and stabilize emulsions. Proteins generally have good emulsifying properties which depend on intrinsic protein properties such as molar mass, hydrophobicity, conformation stability, and charge, and on extrinsic physicochemical conditions such as pH, ionic strength, and temperature

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(*27*). During emulsification proteins adsorb at the oil/water interface of the elongated oil droplets. The adsorbed proteins lower the interfacial tension, thus facilitating droplet breakup, and preventing immediate recoalescence of the droplets (*28*). Once at the interface, proteins are considered to unfold to varying extents, reorient, rearrange, and spread (*29*). The hydrophobic loops orient toward the apolar oil phase, while polar charged segments extend into the aqueous phase (*29*). Once an emulsion is formed various instabilities may occur. Creaming is the rise of droplets to the top of the emulsion due to the density difference between the dispersed and the continuous phases. Droplet aggregation may also occur in emulsions, and may lead to coalescence if the physical film between two droplets is ruptured, resulting in the formation of a single, larger droplet.

In this paper the emulsion-forming and -stabilizing properties of individual sunflower proteins are studied as a function of pH and ionic strength and after heat treatment. These properties are then used to explain the emulsion properties of sunflower isolate (SI) and helianthinin/SFAs mixtures.

MATERIALS AND METHODS

Materials. Dehulled "Mycogen Brand" sunflower seeds were purchased from H.Ch. Schobbers B.V. (Echt, The Netherlands). Tricaprylin oil ($\rho = 0.9540$ kg·dm^{-3}, $n_D = 1.4466$) was purchased from Sigma (Zwijndrecht, The Netherlands). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

Sunflower protein isolate (SI) was obtained as described by González-Pérez and co-workers (*14*). Helianthinin was obtained as previously described (*22*), but with omission of the last gel permeation chromatography step. The resulting helianthinin preparation was mostly in the 11S and 7S forms (90%), next to about 6% in its monomeric form and the presence of other protein impurities (4%). Also a fraction corresponding to the monomeric form of helianthinin was isolated, as described by González-Pérez and co-workers (*22*).

Preparation of SFAs. The defatted dephenolized meal, obtained as described earlier (*14*), was suspended in water [2% (w/v)] and stirred for 2 h while the pH was kept 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 a maximum speed of 3500 rpm. Filter cloths (mesh size 1 μm) were purchased at Lampe technical textiles BV in Sneek (The Netherlands). The pellet was reextracted at similar conditions [2% (w/v), suspension, pH 5.0], and the two 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 molecular mass cutoff of 3 kDa (A/G Technology Corp., Needham, MA) until the conductivity of the retentate remained constant. The retentate obtained was freeze-dried to yield the SFAs preparation with a protein content above 95% (*26*). The resulting SFAs preparation contained about 4% other protein impurities as determined by gel permeation chromatography (results not shown). Tricine SDS−PAGE of the SFAs preparation showed two bands with approximate molecular masses of 12 and 15 kDa (*26*).

Preparation of the Protein solutions. Protein dispersions (5.0−8.0 mg/mL) were prepared from SI, SFAs, and helianthinin in 22 mM Tris−HCl buffer (pH 7.1), 30 mM Tris−HCl buffer (pH 8.0), or 23 mM sodium phosphate buffer (pH 3.0), each having an ionic strength of 20 mM. Protein dispersions were also prepared from SFAs in 30 mM sodium acetate buffer (pH 5). In addition, all dispersions were prepared at an ionic strength of 100 mM using the same buffers containing 80 mM NaCl. The buffer solutions contained a preservative [0.02% (w/v) sodium azide] to inhibit microbial growth.

Mixtures of SFAs and helianthinin were prepared at pH 7.1 (22 mM Tris−HCl buffer) by mixing standard solutions (4.0 mg/mL) of these proteins to obtain protein solutions with 10%, 25%, 50%, and 75% SFAs.

All protein dispersions prepared were stirred overnight at 16 °C, after which the pH was measured and if necessary adjusted with small volumes of NaOH and HCl (0.1−1 M). Next, the protein dispersions were centrifuged (3000*g*, 30 min, 20 °C) and filtered over a 0.2 μm pore size filter (Schleicher and Schuell, Dassel, Germany). The protein concentration of the final protein solutions was estimated using the method of Bradford (*30*) with bovine serum albumin as a standard.

Part of the helianthinin dispersion at pH 3 was adjusted (after 10−15 min kept at the latter pH) to pH 7 and 8 by addition of NaOH (0.1−1 M) and subsequently centrifuged (3000*g*, 30 min, 20 °C). The supernatant was further concentrated with Microcon centrifugal concentrators YM-3000 (Millipore, Etten-Leur, The Netherlands). These treatments are referred to as pH 3 → 7 and pH 3 → 8 treatments, respectively.

Protein samples for testing the effect of heat treatment were prepared by making dispersions of 10.0 mg/mL helianthinin in 30 mM Tris−HCl buffer (pH 8.0). The dispersions were centrifuged (3000*g*, 30 min, 20 °C), and the supernatant was filtered over a 0.2 μm pore size filter (Schleicher and Schuell), and subsequently heated in a thermostated water bath at 65 or 100 °C for 30 min. Heated samples were cooled on ice and centrifuged (3000*g*, 30 min, 20 °C) and the supernatants filtered over a 0.2 μm pore size filter (Schleicher and Schuell). The supernatant resulting from the heat treatment at 100 °C was further concentrated with Microcon centrifugal concentrators YM-3000 (Millipore, Etten-Leur). Finally, 0.2 g/L sodium azide was added to the protein solutions. Part of the 100 °C treated sample was also used at pH 7.

Emulsion Preparation. Emulsions were made by mixing 1 mL of tricaprylin oil and 9 mL of protein solution for 1 min at 11000 rpm with an Ultra Turrax type T-25B (Janke & Kunkel GmbH, Germany). The coarse preemulsion was further homogenized by passing it 10 times at 6 MPa through a Delta Instruments HU 2.0 laboratory scale high-pressure homogenizer (Delta Instruments, Drachten, The Netherlands).

The absence of flocs and/or aggregates was checked by light microscopy at a magnification of 400×. The droplet size was calculated as the volume-surface average diameter (d_{32}) given by $d_{32} = S_3/S_2 = \sum N_i d_i^3/\sum N_i d_i^2$, with N_i and d_i the number and diameter of droplets in size class i, respectively (*31*). The mentioned parameter was estimated using a Coulter Laser LS 230 (Beckman Coulter, Mijdrecht, The Netherlands) immediately after homogenization ($t = 0$ h). When aggregation was detected, the particle size distribution was measured after dilution (1:6 v/v) of the emulsion with 3% (w/v) SDS solution. The instability of the emulsions against coalescence was estimated by measuring the decrease of the turbidity at 500 nm (*32*). For this purpose, the emulsions were diluted (1:100 v/v) in a 0.1% (w/v) SDS solution to stabilize the droplets and to disperse any aggregates present, as monitored with a microscope. Creaming was monitored visually.

To investigate the effect of calcium ions on emulsion properties at pH 7, 8, and 3, a 216 mM $CaCl_2$ solution was added to emulsions prepared at pH 7, 8, and 3 (buffers described above, 4.0 mg/mL protein), resulting in a final Ca^{2+} concentration of 60 mM. Reference samples with the same ionic strength were prepared by adding NaCl. Furthermore, the creaming rate of helianthinin emulsions (10.0 mg/mL protein) at pH 8 after $CaCl_2$ or NaCl addition was monitored using a TurbiScan MA 2000 (Sci-Tec Inc., Worthington, OH). Various amounts of both salts were added, resulting in ionic strengths of 60, 120, 180, and 300 mM. Emulsions were prepared and tested at least in duplicate.

Surface Excess. The surface excess of emulsions was estimated using an indirect depletion method that is based on the estimation of the amount of unadsorbed protein and the interfacial area of the emulsion (*33*). The surface excess (Γ) of emulsions can be determined from the concentration (mg/m^3) of the protein solution before emulsification, the concentration (mg/m^3) of unadsorbed protein, and the specific area of the emulsion (A, m^2/m^3). A can be calculated from $A = 6\varphi/d_{32}$ (*34*), in which φ is the volume fraction of oil in the emulsion. For helianthinin emulsions (pH 7, $I = 50$ mM) the surface excess (Γ) was determined as a function of the protein concentration over the interfacial area of the emulsion (c/A), in which c is the protein

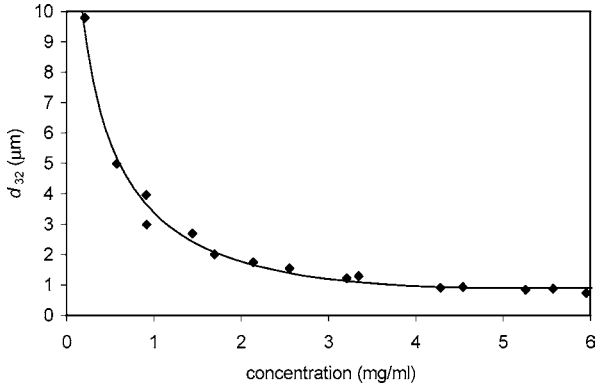

Figure 1. Average droplet diameter (d_{32}) of emulsions made with helianthinin (pH 7, $I = 20$ mM) as a function of protein concentration (mg/mL).

concentration. For these experiments protein concentrations ranging from 0.21 to 6 mg/mL were used. For emulsions made at other conditions, Γ was determined at a single protein concentration. For determination of the concentration of unadsorbed protein, the emulsion droplets were separated from the aqueous phase by centrifugation at 12000*g* for 30 min, resulting in a cream layer and a serum layer. The serum layer was taken and again centrifuged. This procedure was repeated three times, and the final serum was filtered over a 0.2 μm pore size filter (Schleicher and Schuell) and its protein content estimated. The cream layers were dispersed in the buffer solution, keeping the volume fraction of oil equal to that of the original emulsion. The washing buffer obtained after centrifugation (30 min, 12000*g*) of the redispersed emulsion was centrifuged at least two times more and then filtered over a 0.2 μm pore size filter (Schleicher and Schuell) and its protein content determined. This washing procedure was repeated once. The protein concentration was determined using the method of Bradford (*30*) with bovine serum albumin as a standard. The surface excess was calculated as $\Gamma = [\Delta c\ (\mathrm{mg/m^3})]/[A\ (\mathrm{m^2/m^3})]$, where Δc is calculated as $c_{\mathrm{emulsion}} - c_{\mathrm{serum}} - c_{\mathrm{washing\ 1}} - c_{\mathrm{washing\ 2}}$.

Gel Permeation Chromatography. Gel permeation chromatography was carried out to determine the relative amount of helianthinin and SFAs in SI and in the SFAs/helianthinin mixtures. Furthermore, the possible preferential adsorption of sunflower proteins to the oil/water interface in emulsions made with mixtures of SFAs and helianthinin was investigated by comparing the protein composition in the original protein solution to that in the serum. The serum was cleaned from residual oil before injection onto the gel permeation column using the procedure already described for determining the surface excess.

Gel permeation chromatography was performed on an Äkta Explorer System (Amersham, Pharmacia Biotech, Uppsala, Sweden). Samples of 0.2 mL of the protein solutions were applied directly to a Superdex 200 HR 10/30 column and eluted with the buffer solution used to prepare the emulsion, at a flow rate of 0.5 mL/min at room temperature. The absorbance of the eluate was monitored at 214 and 280 nm.

RESULTS

Droplet Size and Surface Excess of Helianthinin Emulsions. The volume-surface average droplet size (d_{32}) of emulsions made with helianthinin (pH 7, $I = 20$ mM) as a function of protein concentration is shown in **Figure 1**. At protein concentrations lower than 1.5 mg/mL, the average size of the oil droplets formed decreased sharply with increasing protein concentration. Above a concentration of about 4 mg/mL, a surplus of protein was present and a more or less constant droplet size (~1 μm) was obtained.

In **Figure 2**, the surface excess of emulsion droplets prepared with helianthinin (pH 7, $I = 20$) is shown. The surface excess is given as a function of protein concentration (c) over specific interfacial area (A) to allow comparison of the surface excess with those of emulsions made with other proteins and different

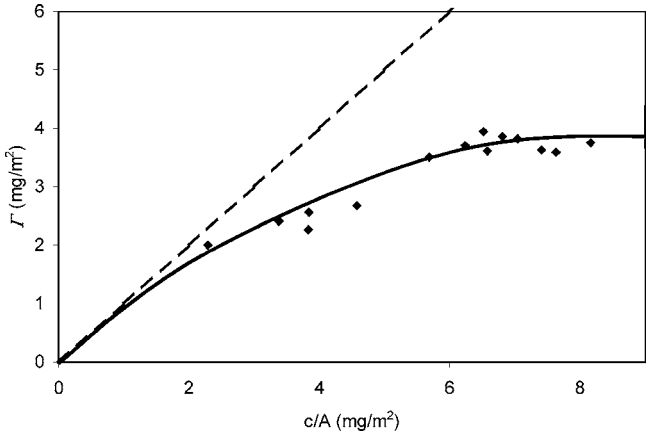

Figure 2. Surface excess (Γ, mg/m^2) of emulsions made with helianthinin (pH 7, $I = 20$ mM) as a function of protein concentration over a specific surface area (c/A, mg/m^2). The maximum possible surface excess at any value of c/A is displayed as a dashed line.

interfacial areas. In **Figure 2**, the maximum possible surface excess at any value of c/A is displayed as a dashed line. At c/A values above 3.0 mg/m^2, the droplet interface became saturated with protein and the experimental curve started to deviate more and more from the theoretical curve. Finally, a plateau surface excess was reached at about 3.6 mg/m^2.

Emulsion Properties of Helianthinin. The emulsion properties of helianthinin were studied at pH 3, 7, and 8. **Table 1** shows the results of these emulsion tests at various pH values. The average standard deviation of the average droplet size, $\sigma(d_{32})$, was estimated as 0.05 μm on the basis of the properties of the emulsions shown in **Table 1**. The accuracy of the Γ values was estimated as described by Oortwijn and Walstra (*33*). This resulted, in the case of SFAs (pH 3, $I = 20$ mM), with $\sigma(d_{32}) = 0.02$ μm, in a $\sigma(A)$ of 0.4 m^2, in which $\sigma(A)$ is the standard deviation of the surface area of 1 mL of separated oil. The other parameters for this emulsion were estimated to be $\Delta c = 1.52$ mg/mL, $\sigma(c) = 0.065$ mg/mL, $A = 10.0$ m^2, $\varphi = 0.1$, and $\sigma(\varphi) = 0.0005$, where Δc is the difference in protein concentration between the original protein solution and that in the serum layer after centrifugation, A is the surface area of 1 mL of separated oil, and φ is the volume fraction of oil in the emulsion. $\sigma(\varphi)$ and $\sigma(c)$ are the standard deviations of φ and Δc, respectively. From these values the standard deviation of Γ was calculated as being 0.07Γ. The average standard deviation of Γ was calculated to be 0.10Γ. On the basis of these calculations, differences in surface excess of less than 10% were considered not to be significant. Further details concerning the calculations can be found in the original publication.

Microscopic studies indicated that part of the oil droplets had formed small aggregates at pH 7 ($I = 20$ mM). Dilution (1:10) of these emulsions in 0.1% SDS solution before microscopic inspection displayed only separate droplets. The average droplet sizes (d_{32}) of emulsions made at pH 7, after dilution of the emulsion in SDS solution, were larger than at pH 3 and 8 ($I = 20$ mM, **Table 1**). All emulsions were stable against creaming for at least 12 h, although emulsions made at pH 8 and 3 were more stable against creaming than emulsions made at pH 7. Emulsions made with helianthinin did not show coalescence at any of the conditions investigated as indicated by the stable turbidity at 500 nm.

Significant differences in Γ were found at the various pH values studied ($I = 20$ mM). The surface excess was relatively low at pH 7, while it was relatively high at pH 8 (**Table 1**), probably due to protein aggregation at pH 8 (*35*).

Table 1. Characteristics of Emulsions Made with Sunflower Protein Preparations

sample	pH	*I* (mM)	*T* (°C)	C_0[a] (mg/mL)	d_{32} (μm)	$\Gamma_{protein}$ (mg/m^2)	droplet aggregation[b]	coalescence[c] (24 h)	creaming[d]
helianthinin[e]	7	20		4.3	1.05	3.5	*	no	S ≈ 12 h
	7	100		3.8	1.04	3.4	***	no	1 h < I
	7	20	100	2.3	0.73	2.3	no	no	S ≈ 18 h
	8	20		4.3	0.76	4.5	no	no	S ≈ 48 h
	8	100		4.8	0.90	4.6	**	no	I ≈1 h
	8	20	100	4.5	0.68	3.9	no	no	S > 48 h
	8	20	65	4.9	0.79	4.2	no	no	S ≈ 24 h
	3	20		4.8	0.91	3.9	no	no	S > 120 h
	3	100		4.9	0.78	4.5	no	no	S > 48 h
	3 → 7	20		2.5	0.83	2.4	no	no	S ≈ 18 h
	3 → 8	20		5.0	0.67	3.7	no	no	S > 48 h
monomer[e]	8	20		3.9	0.65	3.3	no	no	S > 24 h
SFAs	7	20		5.1	1.07		*****	*****	IC
	7	100		5.0	0.97		*****	****	IC
	8	20		4.0	1.20		***	****	IC
	8	100		4.0	0.94		**	**	IC
	5	20		3.9	0.92	1.21	***	no	I < 15 min
	5	100		3.7	0.89	1.32	***	no	I < 15 min
	3	20		4.8	0.60	1.52	no	no	S > 48 h
	3	100		4.6	0.57	1.39	no	no	S > 120 h
SI	7	20		4.9	0.95		***	*	I < 1 h
	7	100		4.7	1.10		****	*	I < 1 h
	8	20		5.1	0.68	3.8	no	no	S ≈ 24 h
	8	100		5.0	1.11		***	*	I < 1 h
	3	20		4.0	0.68	2.5	no	no	S > 48 h
	3	100		4.8	0.73	4.4	*	no	S > 24 h
SFAs/helianthinin mixtures (% SFAs)[f]									
10	7	20		4.2	0.87		***	*	I < 1 h
25	7	20		4.1	0.98		***	**	IC
50	7	20		3.9	0.97		****	***	IC
75	7	20		4.0	0.95		****	****	IC
calcium addition to helianthinin emulsions									
helianthinin[e] + calcium	8	60[g]		10	0.71		*****	no	I ≈ 1 h
	8	120[g]		10	0.71		*****	no	I ≈ 1 h
	8	180[g]		10	0.71		*****	no	I ≈ 1 h
	8	300[g]		10	0.71		*****	no	I ≈ 1 h
	8	120[g]		4.0	0.76		*****	no	I < 1 h
	7	30[g]		4.0	1.00		*****	no	I < 1 h
	3	120[g]		4.8	0.91		no	no	S

[a] C_0 = protein concentration before emulsification. [b] More asterisks indicate increasing size of aggregates, and "no" indicates absence of aggregation. [c] More asterisks indicate a higher extent of coalescence in 24 h, and "no" indicates absence of coalescence. [d] Visual observation of creaming: I, instability (within 1 h); IC, creaming immediately (after emulsion formation); S, stable (after 1 h). [e] "Helianthinin" and "monomer" refer to the helianthinin preparation and the monomeric form of helianthinin, respectively, as described in the Materials and Methods. [f] Proportion of SFAs in the protein mixture. [g] Ionic strength due to $CaCl_2$.

Droplet aggregation was observed at pH 8 upon increasing the ionic strength. At pH 7, droplet aggregation augmented when the ionic strength was increased from 20 to 100 mM (**Table 1**). Independent of the pH, increasing the ionic strength resulted in a lower stability of the emulsions against creaming. Aggregation was most pronounced at pH 7. Increasing the ionic strength resulted in an increase in droplet size at pH 8 and in a decrease in droplet size at pH 3. The ionic strength did not affect Γ at pH 7 and 8, but significantly increased it at pH 3 (**Table 1**).

Heating of the helianthinin solutions at 65 °C (pH 8) and 100 °C (pH 7 and 8) before emulsification resulted in emulsions that did not show droplet aggregation and were stable against coalescence. Heat treatment at 65 °C, however, resulted in emulsions that were less stable against creaming than those made from unheated helianthinin and helianthinin treated at 100 °C.

The pH 3 → 8 and the pH 3 → 7 treatments resulted in emulsions with properties similar to those of the emulsions prepared after helianthinin was heated at pH 8 (100 °C) and at pH 7 (100 °C). These emulsions were characterized by a smaller average droplet size, and the absence of droplet aggregation, compared to the untreated samples. Emulsions prepared with the monomeric form of helianthinin (*22*) (pH 8, I = 20 mM) were similar to emulsions prepared with helianthinin heated at 100 °C. These emulsions did not show droplet aggregation and were stable against coalescence. Their average droplet size was also significantly smaller than for the native multimeric forms of helianthinin (pH 8).

Emulsions Made with SFAs. The emulsion properties of SFAs were studied at pH 3, 5, 7, and 8. The use of SFAs resulted in emulsions that were less stable against creaming than those made with helianthinin, except for emulsions made at pH 3 (**Table 1**). Emulsions at pH 5, 7, and 8 were destabilized by droplet aggregation, which resulted in instant creaming. Especially emulsions made at pH 7 and 8 were unstable against coalescence, as indicated by a drastic decrease in turbidity at 500 nm during the first hours. Interestingly, SFAs formed very stable emulsions at pH 3, especially at high ionic strength. The average droplet size of emulsions made with SFAs at pH 3 was the smallest of all the emulsions tested. Significantly smaller average droplet sizes were also obtained at pH 8 after the ionic strength was increased. The surface excess of SFAs-stabilized emulsions was significantly lower than for helianthinin-stabilized emulsions.

Emulsions Made with SI. The results of the emulsion experiments with SI at pH 3, 7, and 8 are also shown in **Table 1**. Emulsions made with SI at pH 3 were the most stable against droplet aggregation and coalescence, and only little aggregation

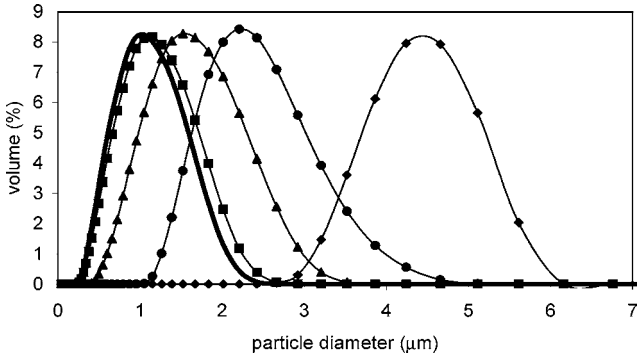

Figure 3. Average size of the (deflocculated) droplets in emulsions prepared with mixtures of helianthinin and SFAs at pH 7 ($I = 20$ mM) just after emulsification (10% SFAs, thick line) and 24 h later for mixtures containing various amounts of SFAs: 10% (■), 25% (▲), 50% (●), and 75% (◆).

occurred upon increasing the ionic strength (100 mM). Although the average droplet size did not change significantly upon increasing the ionic strength at pH 3, a significant increase in surface excess was observed. At pH 7 ($I = 20$ and 100 mM) and pH 8 ($I = 100$ mM), extensive droplet aggregation and a low degree of coalescence resulted in a poor stability of SI emulsions against creaming. At low ionic strength (20 mM), the emulsions made at pH 8 were more stable against creaming and the average droplet size was much smaller than at high ionic strength. Furthermore, at pH 8 ($I = 20$ mM) no aggregation was observed.

Emulsions Made with Mixtures of Helianthinin and SFAs at pH 7. Clear correlations were found between emulsion properties and SFAs content in emulsions made with mixtures of helianthinin and SFAs at pH 7 ($I = 20$ mM, **Table 1**). Droplet aggregation and coalescence occurred in all the emulsions, but both processes were much more extensive for protein solutions containing high amounts of SFAs. **Figure 3**, which shows the particle size of deflocculated (using SDS solution) emulsion droplets made with various proportions of SFAs after 24 h, indicates that coalescence increases with SFAs content. Coalescence occurred in all the cases and was more pronounced for emulsions containing high amounts of SFAs. However, no significant differences in the initial average droplet size were observed for these emulsions (**Table 1**). **Figure 4** displays, as a typical example, the gel permeation chromatogram of both the original protein solution before emulsification and the serum obtained by centrifugation of the emulsion. From this figure it can be observed that the monomeric form of helianthinin was adsorbed readily at the surface of the emulsion droplets. This form of helianthinin was, however, present only in relatively small quantities compared to the oligomeric forms of helianthinin. SFAs are also adsorbed to a high extent as can be deduced from the decreasing area. The 7S and 11S forms of helianthinin were found to adsorb the least readily.

Effect of Calcium and Sodium in Emulsion Stabilities of Helianthinin. The effect of calcium on emulsion properties of sunflower proteins at pH 7, 8, and 3 was also studied. The formation of large aggregates was observed with a microscope at pH 7 and 8 upon $CaCl_2$ addition. Addition of NaCl also resulted in the formation of droplet aggregates. These aggregates were, however, much smaller in size than in the presence of calcium, which considerably delayed the occurrence of creaming. The droplet size was, however, not affected by these salt additions. Addition of an excess of EDTA to the emulsion caused aggregation due to calcium addition, and subsequent homogenization resulted in breakup of the aggregates, whereas in the absence of EDTA aggregation still occurred after homogenization. Emulsions made at pH 3 showed no aggregation upon calcium addition (**Table 1**).

To study the effects of calcium on creaming, increasing amounts of $CaCl_2$ and NaCl were added to stable helianthinin emulsions (10.0 mg/mL) (pH 8) and creaming was monitored as a function of time. No significant differences where found as a function of salt concentration (**Table 1**). As typical examples, **Figure 5** shows the creaming as a function of time at an ionic strength of 60 mM due to the addition of $CaCl_2$ and NaCl. Emulsions creamed slightly faster after calcium addition during the first hours (**Figure 5**). NaCl addition resulted in a higher degree of creaming after 3 days. Also, the time before creaming becomes evident is much longer after NaCl addition than after $CaCl_2$ addition. At ionic strengths below 50 mM droplet aggregation (pH 8) only occurred when $CaCl_2$ was added (**Table 1**) and not when NaCl was added. Furthermore, immediate dilution of the emulsion resulted in separation of the aggregated droplets caused by NaCl addition, but not when calcium was the cause of droplet aggregation. It was also observed that decreasing the protein concentration of the original solution resulted in faster creaming of the emulsion upon salt (NaCl and $CaCl_2$) addition (results not shown).

DISCUSSION

Emulsion Properties of SFAs. Although, in addition to emulsions made with SFAs, also emulsions made with helianthinin showed droplet aggregation, extensive coalescence only occurred in SFAs-stabilized emulsions. Coalescence is rarely the main destabilization process in protein-stabilized emulsions, but it is often induced by droplet aggregation and creaming. The high conformational stability of SFAs (*26*) may facilitate the observed coalescence, since it probably only allows small conformational changes upon adsorption to the interface. Desorption from the interface is likely to occur when the conformational changes on adsorption are small (*36*), and therefore, the formation of surface tension gradients may be impaired. Droplet aggregation and concomitant coalescence in emulsions made with SFAs could only be avoided at pH 3. The isoelectric range covered by SFAs is about pH 6−10 (*23*, *24*, *37*−*39*). It, therefore, appears that electrostatic repulsion at pH 3 is strong enough to prevent droplet aggregation. Furthermore, at pH 3 the repulsion of charged segments is maximized, which may significantly increase conformational flexibility and thus facilitate more extensive unfolding of SFAs upon adsorption.

The surface excess of SFAs-stabilized emulsions was significantly smaller than that of helianthinin-stabilized emulsions. These results are in accordance with the finding that the surface excess of emulsion droplets is mainly determined by the conformational stability of proteins and the presence of aggregates (*35*).

Emulsion Properties of Helianthinin. In helianthinin-stabilized emulsions, lowering the pH from 8 to 7 and increasing the ionic strength from 20 to 100 mM reduced the electrostatic repulsion and favored droplet aggregation (**Table 1**). The high surface excess at pH 8 is probably due to the formation of protein aggregates, as also observed by gel permeation chromatography. Generally, the surface excess varies between 1.0 and 3.0 mg/m^2 (*35*), but when protein aggregates are adsorbed, it can be higher than 5.0 mg/m^2 (*40*). Despite the occurrence of protein aggregation, droplet aggregation did not occur at pH 8 ($I = 20$ mM).

Effect of Protein Unfolding on the Emulsion Properties of Helianthinin. At pH 3, helianthinin dissociates into mono-

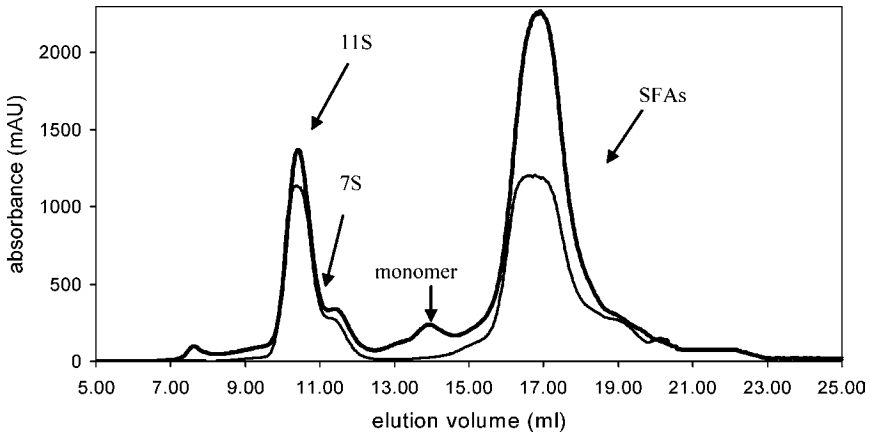

Figure 4. Gel permeation chromatography of a protein solution containing about 80% SFAs and 20% helianthinin at pH 7 (I = 20 mM). The thick line stands for the protein solution before emulsification and the thin line for the protein solution in the serum layer. The absorbance is monitored at 214 nm and expressed in milliabsorbance units (mAU).

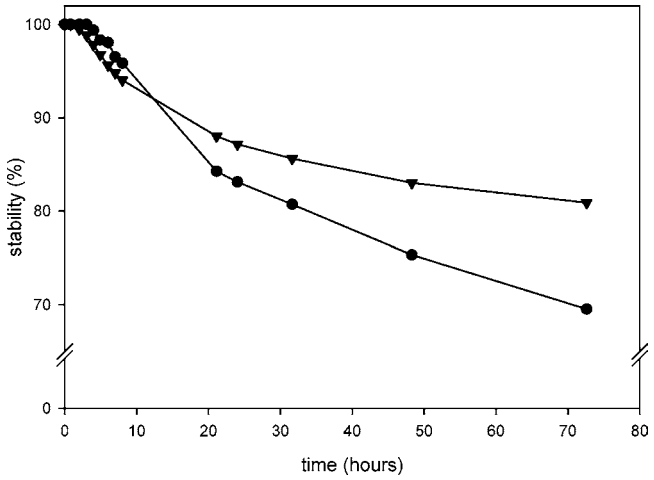

Figure 5. Creaming stability of helianthinin emulsions (pH 8, I = 20 mM) after addition of $CaCl_2$ (▼, 20 mM final concentration) or NaCl (●, 60 mM final concentration). Stability (%) = volume of emulsions without phase separation (i.e., 100% when no phase separation has occurred and 75% when 25% is serum).

mers and loses its tertiary and most of its secondary structure (*22*). These structural changes have a positive effect on emulsion stability at pH 3. In addition, the increased emulsion stability is also observed in emulsions formed with helianthinin solutions that have been treated at pH 3 and then readjusted to pH 7 and 8, most likely because changes in the structure of helianthinin due to low pH are irreversible (*22*). Changes in conformation may also be the reason for the improvement of the emulsion stability by heating helianthinin solutions at 100 °C prior to emulsification. Improvement of emulsion properties of proteins by treatments that induce conformational changes and/or its flexibility has been previously reported (*40*−*43*).

Effect of Calcium on Droplet Aggregation. The specific effect of calcium becomes apparent at relatively low concentrations (17 mM), which correspond to an ionic strength (50 mM) at which NaCl has no effect. Therefore, the formation of specific calcium cross-links between the carboxylic groups of proteins adsorbed at different oil droplets seems very likely. Moreover, at pH 3 aggregation was not observed, because the calcium bridges cannot be formed due to protonation of the carboxylic acid groups.

Emulsion Properties of Protein Mixtures. Synergetic or antagonistic effects on emulsion properties have been reported when proteins differing in their intrinsic properties (molecular size, pI, conformational stability, etc.) were mixed (*44*, *45*). The reconstitution experiments showed, however, an additive effect of helianthinin and SFAs, i.e., decreased stability when increasing proportions of SFAs were added to protein mixtures. The presence of only 10% SFAs in the protein mixture already caused significant coalescence at pH 7. However, at pH 8 (I = 20 mM), where the soluble fraction of SI is estimated to contain about 10% SFAs, a stable emulsion was obtained. The percentage of SFAs in the soluble fraction of SI at pH 7 (I = 20 mM) was estimated to be approximately 20−30%, which is consistent with the properties observed for emulsions made with mixtures having this composition (**Table 1**).

Summarizing, sunflower proteins were shown to form stable emulsions, with the exception of SFAs at alkaline and neutral pH values. Therefore, application of sunflower proteins in food emulsions would preferably be done at acidic pH. Treatments that increase conformational flexibility are shown to improve the emulsion properties, provided they do not lead to extensive protein aggregation and precipitation.

ABBREVIATIONS USED

SFAs, sunflower albumins; SI, sunflower isolate; Γ, surface excess; A, specific area; φ, volume fraction of the dispersed phase; d_{32}, volume-surface average diameter; ANFs, antinutritional factors; I, ionic strength; pI, isoelectric pH; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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