Functionality of the 2S Albumin Seed Storage Proteins from Sunflower (*Helianthus annuus* L.)

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The emulsifying and foaming properties of the 2S albumins of four cultivars of sunflower were studied. Kinetics of flocculation creaming, resistance to coalescence, foam formation, and stability were determined. Results obtained indicated a cultivar dependence on emulsion stability. The proteins associated with emulsion stability were the most hydrophobic, as determined by RP-HPLC, and correspond to a group of methionine-rich 2S albumins previously characterized. Conversely to 2S albumins from other species, those from sunflower produced foams with little or no stability.

Keywords: *Sunflower; 2S albumin*

INTRODUCTION

Sunflower seed contains two major protein classes, the 11S globulins (helianthinins) and 2S albumins (S = sedimentation coefficient $s_{20,w}$). The major 11S globulins have M_r ~300 000 (Schwenke *et al.*, 1979) and consist of a hexamer of subunit pairs, the latter consisting of an α-polypeptide (M_r 30 000−40 000) and a smaller β-polypeptide (M_r 23 000−27 000) linked by disulfide bonds (Dalgalarrondo *et al.*, 1984). The 2S albumins occur in a botanically diverse range of species and usually constitute some 20−50% of the total seed proteins. They have M_r 14 000−19 000, with high amide and cysteine contents (Youle and Huang, 1981). Available gene sequences indicate that they consist of between 100 and 140 amino acid residues, with eight cysteine residues (Allen *et al.*, 1987). In addition, some albumin components contain up to 16 mol % methionine residues (Kortt *et al.*, 1991). The sequences of 2S albumins from a range of species show high sequence similarity and also belong to a large superfamily of proteins which include cereal prolamins and enzyme inhibitors (Kreis *et al.*, 1985). The 2S albumin of most species, for example, Brazil nut (Sun *et al.*, 1987) and oilseed rape (Ericson *et al.*, 1986), consist of two chains linked by disulfide bonds; however, in sunflower 2S albumins consist of a single polypeptide chain (Allen *et al.*, 1987; Kortt *et al.*, 1991; Anisimova *et al.*, 1995). All 2S albumins are derived from larger precursor proteins, and in most cases, they are processed post-translationally to give single monomeric (sunflower) or heterodimeric (*e.g.*, oilseed rape) proteins. However, in sunflower (Allen *et al.*, 1987) and castor bean (Irwin *et al.*, 1990), two such monomers or heterodimers, respectively, may be encoded by a single mRNA and synthesized as a large preproprotein.

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Functional properties so far have been reported on sunflower flours and protein concentrates. These have indicated good emulsification properties (Lin *et al.*, 1974; Huffman *et al.*, 1975; Sosulski and Fleming, 1977; Sosulski, 1979) and good foaming/foam-stabilizing properties (Lawhon *et al.*, 1972; Lin *et al.*, 1974; Canella, 1978; Holm and Breedon, 1983; Kabirullah and Wills, 1988). Functional tests have also indicated a cultivar dependence (Canella, 1978; Holm and Breedon, 1983) which may be related to variability in the protein composition. The present paper reports the functional properties of the 2S albumin fractions from four sunflower hybrid varieties and identifies the protein fractions involved in functionality.

MATERIALS AND METHODS

2S Albumin Purification. 2S albumins were purified from the hybrid sunflower variety Sunbred 254 (Twyfords Seeds U.K. Ltd.) and the three inbred lines Vir104, Vir111, and Vir130 (Vavilov Institute, St. Petersburg, Russia) by a modification of the method of Kortt and Caldwell (1990). Seed was mechanically dehulled, the kernels were crushed and extracted three times with acetone (10:1, v/w, acetone:seed) in a Waring blender, and the resulting concentrate was air-dried. The protein concentrate was then extracted twice with petroleum ether (60−80 °C) (10:1, v/w, solvent:concentrate) by mechanical stirring for 1 h at room temperature, the solvent decanted off, and the concentrate air-dried. The concentrate was extracted with 0.5 M NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1 M Tris/HCl, pH 7.8 (10:1, v/w, buffer:concentrate), by mechanical stirring for 2 h at room temperature. The slurry was centrifuged (13500*g*, 30 min at 20 °C). The supernatant was then cooled to 0 °C and cold methanol added to give a final concentration of 60% (v/v) methanol. The precipitate containing the globulins was recovered by centrifugation (13500*g*, 30 min at 2 °C). The albumins were precipitated from the supernatant by the addition of 3 vol (v/v) of acetone at −20 °C for 16 h and recovered by centrifugation (13500*g*, 30 min at 2 °C). The precipitated albumins were dissolved in water and dialyzed against water using a low cutoff membrane (SpectraPor 3, Pierce and Warriner) and freeze-dried.

Emulsification Properties. Emulsions were prepared at 0.5 and 1.0 mg of protein/mL in 0.1 M sodium phosphate buffer, pH 8.0, using hexadecane as the oil phase.

Measurement of Creaming Flocculation. The protein solution (25 mL) and hexadecane (15 mL) were poured into a

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cylinder containing two electrodes at the base. The conductivity of the protein solution was measured before mixing; then the emulsion was formed using a Polytron PT3000 stirrer operated at 20 000 rpm for 30 s. Droplets formed by the Polytron have a diameter of about 10 μm. The volume of the aqueous phase was continously monitored by conductivity measurement (Loisel and Popineau, 1993). Results are reported as the volume fraction of dispersed oil in the emulsion, calculated from

$$\phi = 1 - [25/15 \times (1 - C_{tc}/C_{sol})]$$

where C_{sol} is the conductivity of the protein solution before emulsification and C_{tc} is the conductivity of the emulsion at time t; 25 and 15 are the volumes of the aqueous and oil phases in milliliters, respectively. Data are reported as a plot of ϕ vs time (min). Kinetic parameters, k_1 and k_2, the rates of destabilization of the emulsion, were measured between initial ϕ and $\phi + 0.1$ and between $\phi + 0.1$ and $\phi + 0.2$, respectively.

Measurement of Resistance to Coalescence. A modification of Graham's (1976) technique was used to measure emulsion stability (Dagorn-Scaviner *et al*., 1987). The emulsion was prepared as described above. To mimic aging, emulsions were centrifuged at 1000*g* for 5 min, and the volume of the separated oil phase was measured at times between 0 and 100 min. The results are expressed as a plot of oil recovered vs time. Experiments were repeated in duplicate.

HPLC. The 2S albumins were separated by RP-HPLC on an analytical Nucleosil C18 (25 × 0.39 cm) 5 μm particle size and 300 Å pore size column at 45 °C, with an increasing gradient of acetonitrile of 20−50% over 45 min. Solvents: A, water + 0.1% trifluoroacetic acid (TFA); B, acetonitrile + 0.08% TFA. Detection was at 220 nm, flow rate 1.0 mL/min.

The distribution of 2S albumins between the aqueous and emulsion phases was determined by HPLC after 5 and 48 h. Samples from the aqueous phase were directly analyzed. The emulsion was washed with buffer (0.1 M sodium phosphate buffer, pH 8.0) to remove proteins in the continous liquid phase of the emulsion, termed the interstitial phase. The washed emulsion was then frozen in order to break the emulsion to a two-phase system, a major oil fraction and a minor aqueous one, the latter phase containing the adsorbed (interfacial) proteins.

Foaming Properties. The foaming properties at 2.0 and 3.0 mg of protein/mL in 0.05 M sodium phosphate buffer, pH 7.8 and 8.0, with a range of salt concentrations from 0 to 0.1 M, were measured as described by Guillerme *et al*. (1993) and Loisel *et al*. (1993). The foam was formed in a plastic column (20 × 3 cm) by blowing air through a porous metal disk (pore size 2 μm). The column contained a pair of electrodes to measure the volume of the liquid (a measure of drainage), the inverse of the resistance being proportional to the volume of the liquid. Protein solution (15 mL) was placed on the disk, bubbling started at a rate of 15 mL/min, and the foam allowed to reach a volume of 50 mL. The level of the foam was measured by a uniline CCD camera (Schmersal, model M-1024). The bubbling was stopped and the foam analyzed for 20 min. To avoid liquid passing back through the disk, an antagonistic pressure was applied. The quantity of liquid at the bottom of the column was continuously monitored, with foam density and foam volume being measured with time. These parameters characterize foaming capacity and stability.

RESULTS

Characterization of 2S Albumin Fractions. Total albumin fractions of the four lines were analyzed by SDS−PAGE, to determine purity, which showed about 8−10 individual bands in each line, with M_rs (under reducing conditions) of 10 000−18 000 (Figure 1, tracks 4−7). Differences in the mobilities and properties of the individual components were also observed between the four lines. The 2S albumin fractions were free from contamination with globulins (which had M_rs of 28 000−40 000; Figure 1, track 3), but some minor bands of lower M_r were present. HPLC analysis indicated a number of different components, differing in their retention times (a measure of surface hydrophobicity) (Figure 2). The albumins were divided into four groups on the basis of their retention; up to 13 peaks were observed in the various fractions (Figure 2).

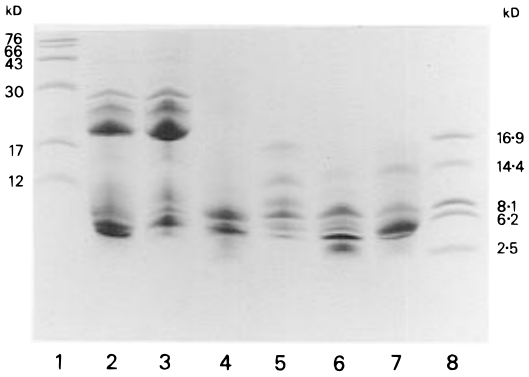

Figure 1. SDS−PAGE of sunflower seed storage proteins. Tracks: 1 high-M_r markers; 2, total Sunbread 254 seed proteins; 3, Sunbread 254 globulins; 4, Sunbread 254 albumins; 5, Vir104 albumins; 6, Vir111 albumins; 7, Vir130 albumins; and 8, low-M_r markers.

Stability of Emulsions. Figure 3 shows the development of flocculation creaming of the 2S albumins of Vir104 and Vir111 at 0.5 and 1.0 mg/mL. The kinetics of emulsion destabilization due to flocculation creaming are reported in Table 1.

At 0.5 mg/mL, the rate constant k_1 was higher for Vir104 and Vir130, indicating a higher initial rate of flocculation creaming than Sunbred 254 and Vir111; the same order was observed for k_2. After 4.5 h the ϕ values of Sunbred 254 and Vir104 were lower than those of Vir111 and Vir130. Furthermore, the former had reached quasi-equilibrium, whereas the latter continued to phase separate. The state of the flocculation creaming process is characterized by the values of the volume fraction of the apolar phase in the creamed phase, a measure of the closeness of packing of the droplets. Assuming that the stirring conditions produced a monodisperse suspension of spherical undeformable droplets 10 μm in diameter, the volume fraction ϕ at equilibrium should be 0.74 (Dagorn-Scaviner *et al*., 1987). The lower values for Sunbred 254 and Vir104 indicate higher repulsion between droplets leading to a higher water retention by the creamed phase than Vir111- and Vir130-coated droplets. This effect of the adsorbed proteins on the hydration of the creamed phase is not linked with the kinetics of the early stages of the development, the k_1 and k_2 values showing differences that are, apparently, not related to the final ϕ values. k_1 and k_2 values are related to flocculation and decantation of droplets, which mainly depend on electrostatic properties of the interfacial layer and steric effects, whereas ϕ is mainly ruled by the hydration capacity of the layers.

The differences in k_1 and k_2 between lines are much smaller at 1.0 mg/mL, and the ϕ after 4.5 h values are similar. At these concentrations, the effects of the differences in 2S albumin fractions on emulsion stability were reduced. This indicates that, at 0.5 mg/mL, the characteristics of the protein fractions were the main determinants of emulsion stability. Stabilization of an emulsion by protein depends on both the surface properties of the components and their concentration. In this case, increasing concentration up to 1.0 mg/mL weakens the effect of the different physicochemical

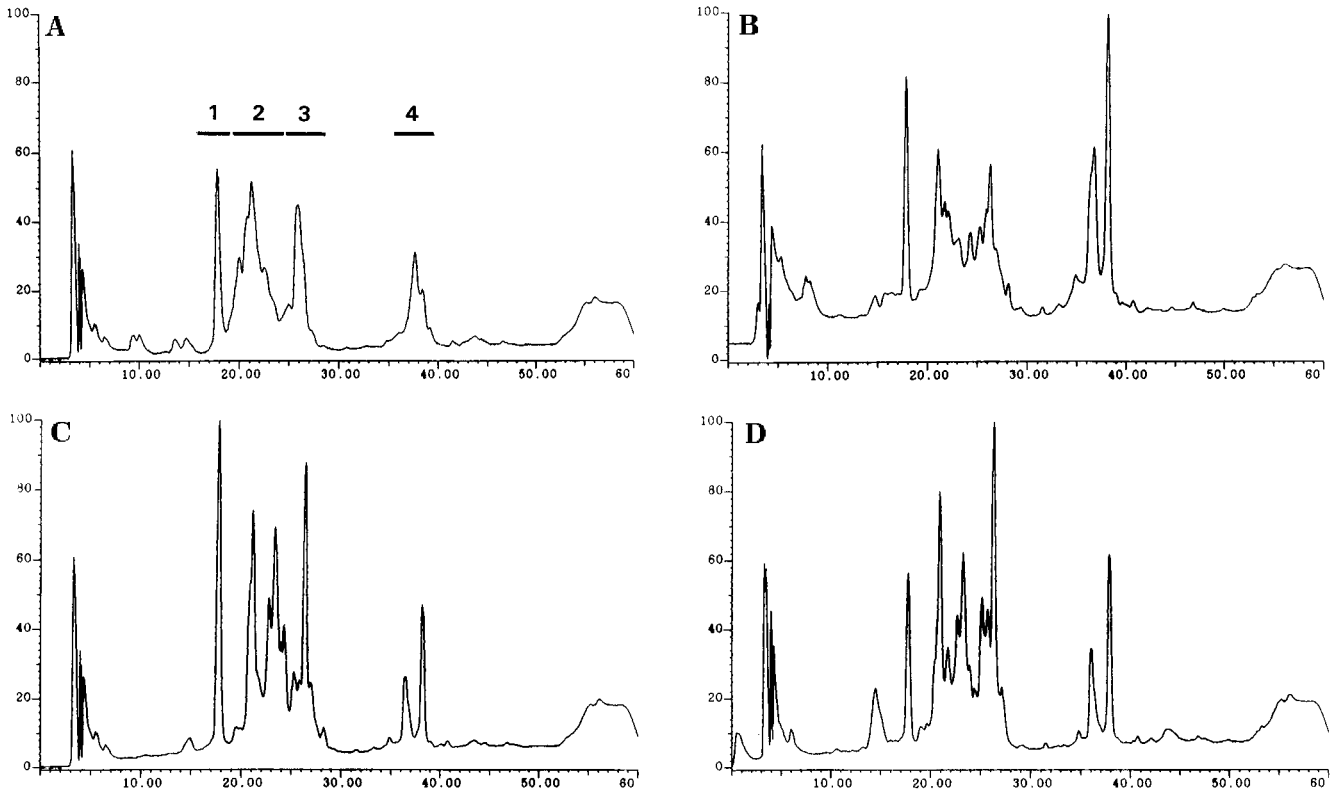

Figure 2. RP-HPLC profiles of albumins of (A) Sunbread 254, (B) Vir104, (C) Vir111, and (D) Vir130. Group 1−4 albumins are marked on trace A.

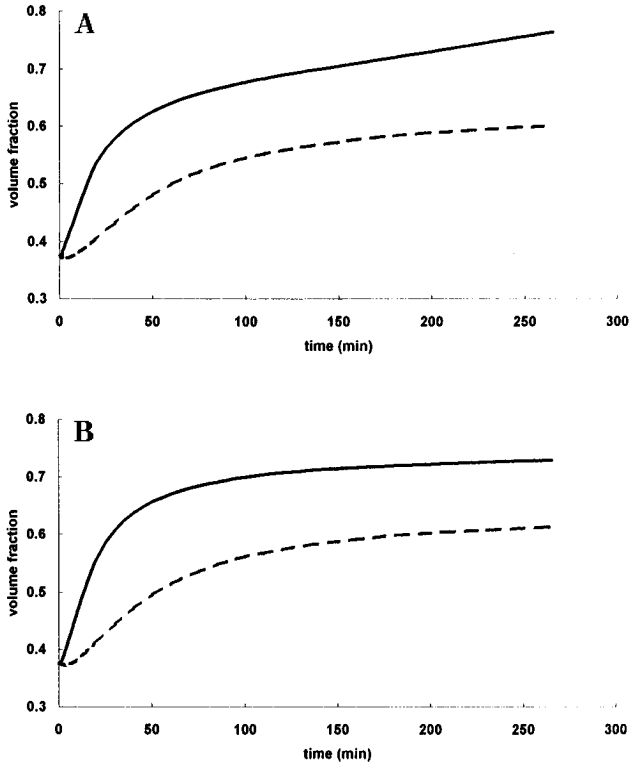

Figure 3. Flocculation creaming of (A) Vir111 and (B) Vir104: (− − −) 0.5 and (—) 1.0 mg/mL.

properties of the various 2S albumin fractions. It can be assumed that surface-active molecules are in excess at 1.0 mg/mL, irrespective of the origin of the 2S albumin fraction. The differences in emulsification properties of the four fractions were not obviously related to differences in protein composition as revealed by SDS−PAGE (Figure 1) or RP-HPLC (Figure 2).

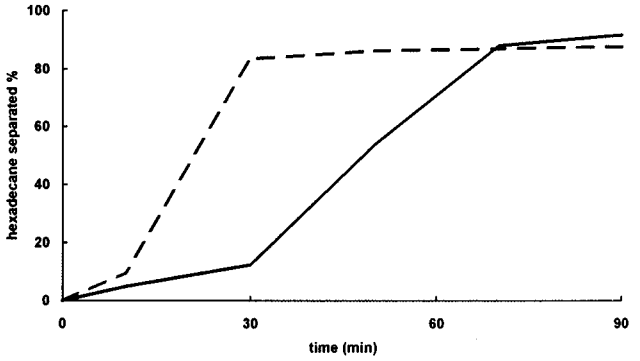

Figure 4. Resistance to coalescence of albumins from Vir104 (—) and Vir111 (− − −).

Table 1. Emulsifying Properties of 2S Albumin Fractions[a]

	0.5 mg/mL			1.0 mg/mL		
hybrid	k_1	k_2	ϕ	k_1	k_2	ϕ
S254	3.9	1.8	0.71			
Vir104	8.4	2.3	0.73	1.6	0.4	0.61
Vir111	5.9	1.8	0.76	1.3	0.3	0.60
Vir130	8.2	2.5	0.82			

[a] k = destabilization rates, 10^{-3} min^{-1}. ϕ = volume fraction of hexadecane in creamed phase after 4.5 h (ϕ_e from geometric theory = 0.74).

Resistance to Coalescence. Figure 4 shows the results obtained for the stability of emulsions prepared with the 2S albumin fractions of Vir104 and Vir111, with percent hexadecane separated plotted vs time. The fractions from lines Vir111 (Figure 4) and Vir130 (data not shown) showed a rapid release of alkane from the emulsion, while those from the other two lines, Vir104 (Figure 4) and Sunbred 254 (data not shown), produced more stable emulsions. The data indicate differences

Table 2. Distribution of 2S Albumins Recovered in Lower Aqueous Phase[a]

	Vir104	Vir111	Vir130	S254
P1	77	71	84	70
P2	44	33	50	33
P3	19	17	25	0
P4	0	0	0	0

[a] Results expressed as percentage (vs initial protein) recovery of 2S albumin fractions from the aqueous phase after 4.5 h.

in the mechanical resistance of the interfacial films over the surface of the oil droplets. The droplets are pushed together and deform during centrifugation, resulting in phase separations if the interfacial film is not strong enough to prevent coalescence.

These results, together with the data from Table 1, indicate that the 2S albumin fractions from Vir104 and Sunbred 254 form more stable emulsions than those from Vir111 and Vir130. To determine whether specific components of the 2S albumins in fractions were involved in emulsion formation and stability, emulsion phases were analyzed by RP-HPLC. Emulsions were prepared from the fractions and allowed to equilibrate for 5 h. RP-HPLC of the initial protein solutions showed four groups of components differing in their retention time (surface hydrophobicity) (Figures 2 and 5). Similar analyses of the aqueous phases collected under the creamed phase of the emulsions showed that they were enriched in the more hydrophilic group 1 and 2 albumins, with lower contents of the group 3 and no group 4 albumins (Table 2).

The analysis was therefore extended to emulsions aged for 5 and 48 h, with the analysis of aqueous, interstitial, and adsorbed phases. The results for Vir130 are shown in Figure 5 and the data for the aqueous and interstitial phases in Table 3. It was not possible to analyze the proteins associated with the interfacial film. This was because freezing of the washed emulsion failed to break the surface film, with the protein remaining associated with the alkane. These results demonstrate that the most hydrophobic group, group 4, was most effective at stabilizing emulsions, a small proportion of which was recovered in the interstitial phase; most remained in the interfacial layer (Table 3).

Foaming Properties. Foams produced from the total 2S albumins of the four varieties rapidly degraded, showing little or no stability (data not shown). Similarly, foams prepared from group 4 albumins, which were most effective at stabilizing emulsions, also showed no foam stabilization (data not shown). Foaming studies were therefore not pursued.

DISCUSSION

The emulsifying and foaming properties of a protein depend on a number of factors but predominantly on the ability to decrease surface tension by adsorption at an oil/water or air/water interface, with unfolding to expose hydrophobic and hydrophilic residues to their preferred phase. The properties of the interfacial layer (*i.e.*, electrostatic, structural, and mechanical) are crucial in determining emulsification and foaming behaviors. In emulsification, they oppose flocculation and creaming and prevent coalescence of droplets, leading to phase separation, whereas in foams, they confer the ability to retain moisture and withstand mechanical shock and aging changes (Halling, 1981; Dickinson, 1986; Kinsella and Phillips, 1989; Phillips *et al.*, 1990).

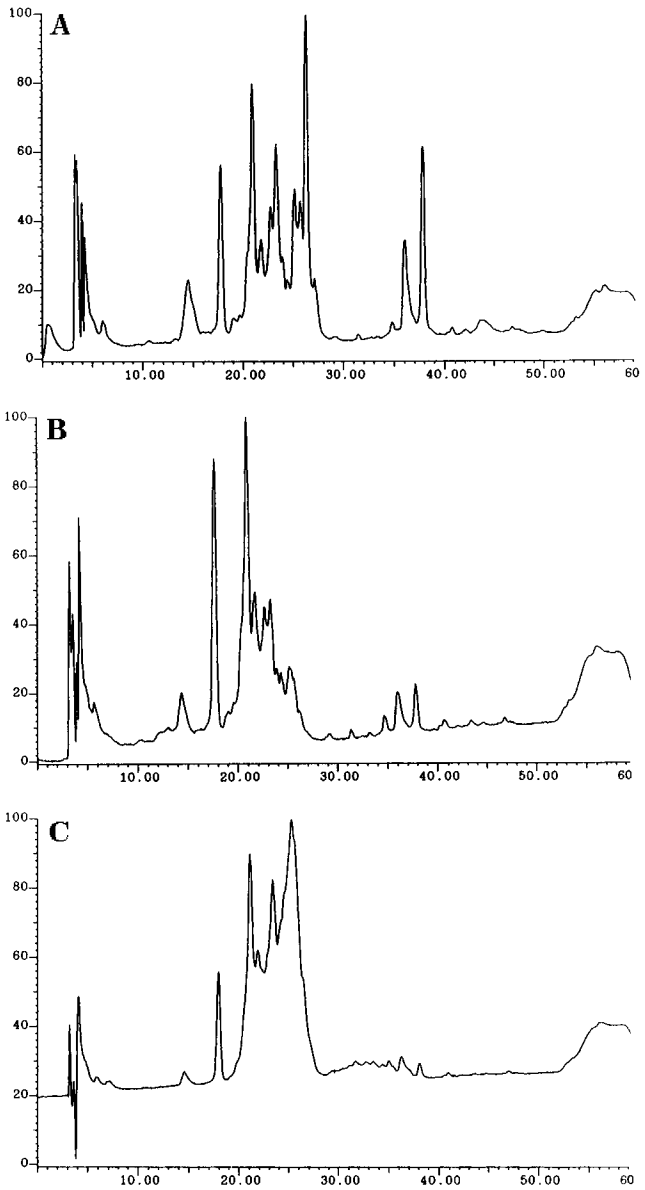

Figure 5. RP-HPLC traces of albumin fractions recovered from emulsion phases of Vir130: A, initial protein solution; B, aqueous phase; and C, interstitial phase.

Table 3. Distribution of 2S Albumins in the Aqueous and Emulsion (Interstitial and Adsorbed) Phases[a]

	Vir104		Vir111		Vir130		Sun254	
time (h)	5	48	5	48	5	48	5	48
aqueous	25	32	31	52	37	55	25	37
int		35		16		26		33
emulsion	75		69		63		75	
ads		33		32		19		30

[a] Results expressed as percent total protein.

Total 2S albumin fractions from sunflower seeds are composed of a mixture of proteins, with similar M_rs (Figure 1) but differing widely in their hydrophobicities, as observed by RP-HPLC (Figure 2). This indicates that they may also differ in their surface properties in systems, such as foams and emulsions.

Emulsification studies showed that the 2S albumin fractions of some hybrids were more active than others, although it was not possible to relate the emulsification properties to compositional differences. However, analyses of the protein compositions of the different phases (aqueous, interstitial, and adsorbed) of the emulsions

after flocculation and creaming indicated that the most hydrophobic 2S albumins are the most active in emulsion stabilization, being either present in the interstitial phase or strongly adsorbed to the hexadecane droplets. The group 4 albumins correspond to the proteins called SFA-8 by Kortt and Caldwell (1990). Direct amino acid sequencing and a corresponding cloned cDNA showed that these consist of 103 residues, with 16 methionine and eight cysteine residues (Kortt *et al.*, 1991). The protein would, therefore, be predicted to have a tightly folded structure, as would other members of the 2S albumin group, which have similar numbers of cysteine residues (Kortt and Caldwell, 1990). Correlative studies have shown that an increase in surface hydrophobicity, rather than overall hydrophobicity, is related to a lowering of interfacial tension and emuslifying capacity (Dickinson, 1986). This relationship appears to hold for the 2S albumins of sunflower, those with higher surface hydrophobicity, as measured by their retention on a RP-HPLC column, being associated with the interstitial and adsorbed phases of the emulsion (Table 2).

The 2S albumins lack any foaming activity under the conditions studied and do not contribute to foam formation or foam stabilization. Electrostatic repulsion is weakest at the isoelectric point of a protein, and providing the protein remains soluble, globular proteins generally have good foaming properties (Kinsella, 1981). The calculated p*I* of SFA-8 (calculated from the sequence of Kortt *et al.*, 1991) is around 7.5, which is close to that used for the analyses (pH 7.0 and 8.0) and at a range of salt concentrations. Limited sequence information of other sunflower 2S albumins and the data of Youle and Huang (1981) would indicate p*I*s in the region of 9−10, so that experimental conditions were near to those shown to be optimal for foaming properties. The lack of foam formation or foam stabilization ability is unusual, as most proteins exhibit at least some foaming properties. The solvent extractions used to prepare the fractions are unlikely to have markedly affected the foaming properties, as similar conditions have been shown to have little effect on the functional properties of flours or concentrates of sunflower (Rahma and Rao, 1981; Holm and Breedon, 1983). Huffman *et al.* (1975) found that stable foams were formed with concentrates below pH 5 and above pH 7, while Sosulski and Fleming (1977) showed that the foaming was related to nitrogen-extractability and that foaming followed the nitrogen-extractability profiles of flours and concentrates. The poor foaming properties of the sunflower 2S albumins contrast with those of the 2S albumins of other species which have been shown to possess good foaming activity (Nitecka *et al*., 1986; Schwenke *et al*., 1991). Sunflower 2S albumins are unique among the 2S albumins characterized so far in that they consist of a single polypeptide chain (Anisimova *et al*., 1995), whereas other 2S albumins are proteolytically cleaved to give two polypeptide chains joined by disulfide bonds. The sunflower 2S albumins may therefore be more structurally rigid than other 2S albumins, which could, in turn, be responsible for the failure to initiate or support foam stabilization. The data would therefore indicate that the foaming capacity of sunflower flours and concentrates reported by other workers (Canella, 1978; Holm and Breedon, 1983; Kabirullah and Wills, 1988) is due to the 11S helianthinin components (Kim and Kinsella, 1987a,b; Yu and Damodaran, 1991).

The contrasting functional properties of the 2S albumins of sunflower and other species make then ideal model systems for exploring the molecular bases for the ability of proteins to stabilize foams and emulsions.

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