Characterization of *Helianthus annuus* L. Storage Proteins

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A rapid method is described for isolating the globulin fraction (helianthinin) of sunflower seeds (*Helianthus annuus* L.). The protein was characterized by chromatography and electrophoresis and compared with the globulin extracted from protein bodies. The native protein shows an apparent molecular weight of 300K, but, alternatively, a 190K band is present in some selfed lines of sunflower. Electrophoresis under denaturing conditions evidences four major components with molecular weights 39.2, 32.5, 25.6, and 23.2K, each containing two polypeptides and some minor polypeptides. In the 190K protein, the 32.9K component is absent. Isoelectric focusing indicated an heterogeneity of subunits within a p*I* range of 5.0–6.2 as confirmed by two-dimensional electrophoresis.

Seed reserve proteins of sunflower (*Helianthus annuus* L.) are globulins and albumins stored in protein bodies (Buttrose and Lott, 1977). The globulin fraction amounts to 80% of the total proteins (Baudet and Mossé, 1977) and is composed of one major globulin called helianthinin, which is an 11S protein with an oligomeric structure and a molecular weight of 300K (Schwenke et al., 1974).

Several authors proposed different procedures for the extraction and purification of this protein, the characterization of which was carried out through chromatographic and electrophoretic techniques.

Baudet and Mossé (1977) separated the globulin fraction through Sephadex G-200 chromatography and obtained five peaks; the major component had a molecular weight of 43K. By contrast, Sabir et al. (1973) found as the most abundant peak a 32–34K polypeptide. Reichelt et al. (1980) investigated the quaternary structure of helianthinin. They observed an esameric protein consisting of six spherical subunits Dalgalarrondo et al. (1985) studied the subunit heterogeneity of the globulin fraction and supposed the existence of structural similarities between helianthinin and legumin, the major globulin of *Pisum* and *Vicia* seeds.

In the present paper, a new rapid method for the extraction of sunflower seed globulins is proposed by which these proteins can be extracted from a little portion of each seed without contamination of albumins and chlorogenic acid. The characterization of globulins extracted with this technique was carried out and the results were compared with those reported by other authors. Finally, variations in polypeptide subunits are examined by electrophoretic analyses of globulins extracted from seed of selfed lines.

MATERIALS AND METHODS

Isolation of Seed Globulin. Seed samples of *H. annuus* L. (cv. Argentario) were decoated, and oil was extracted with an overnight treatment in *n*-hexane (seed to solvent ratio of 1:10, w/v) with continuous stirring. The material was then recovered by filtration and lyophilized. The seeds were homogenized at 4 °C with 25 mM citrate–phosphate buffer (pH 5.0) containing 2 mM 2-mercaptoethanol. The slurry was centrifuged at 20000*g* for 15 min. The precipitate was suspended in the same buffer and centrifuged at 20000*g* for 10 min; this treatment was repeated several times until complete disappearance of chlorogenic acid in the supernatant as judged by spectrophotometric measurements. The precipitate was suspended in distilled water and centrifuged; the pelleted globulins were solubilized in 25 mM borate buffer (pH 8.6) containing 10% NaCl. After centrifugation at 20000*g* for 20 min, the supernatant was collected and used for further analyses.

Storage proteins were also extracted from protein bodies isolated according to Pusztai et al. (1977). The flour was homogenized with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA and 20% sucrose. After filtration through a nylon filter (0.2-mm pore size), the slurry was centrifuged at 1000*g* for 15 min. The precipitate and the superficial lipidic layer were discarded, and the supernatant was centrifuged at 20000*g* for 20 min. The pellet containing the protein bodies was recovered, and proteins were isolated after solubilization in 20 mM borate buffer (pH 8.6) containing 10% NaCl.

Chromatography. Protein solutions were applied to a Sepharose G-50 (Pharmacia) column (2.5 × 67 cm), which had been equilibrated with 25 mM sodium phosphate buffer (pH 7.5). Elution was carried out with the same buffer at a 72 mL/h flow rate. Fractions of 5 mL were collected in an automatic fraction collector, and the absorbance of the eluate was continuously monitored at 280 nm with a linear recorder. The pooled fractions containing globulins were used for a subsequent ion-exchange chromatography on DEAE–cellulose (Cellex-D high-capacity anionic form; Bio-Rad) column (1.0 × 25 cm), which had been equilibrated with 25 mM sodium phosphate buffer (pH 7.5). The elution was carried out with a linear gradient of NaCl (from 0.0 to 0.5 M) in the same buffer at a 30 mL/h flow rate. Fractions of 5 mL were collected, and the absorbance was monitored as above.

Gel Electrophoresis. Gels (7%) for polyacrylamide gel electrophoresis (PAGE) in undenaturing conditions were prepared according to Davis (1964) in a vertical slab appartus. Electrophoresis was performed in 25 mM Tris–glycine buffer (pH 8.3) at 140 V. The gels were stained with 0.2% Coomassie brillant blue in 50% methanol and 7% acetic acid for 1 h and destained with a solution containing 20% isopropyl alcohol and 7.5% acetic acid. When necessary, the gels were also stained with Silver Stain (Bio-Rad).

The electrophoresis gels (12%) in denaturating conditions (SDS-PAGE) were prepared according to Laemmli (1970). Protein samples were diluted 1:4 (v/v) with a solution containing 62.5 mM Tris-HCl (pH 8.3), 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol and the solutions heated at 100 °C for 5 min. Molecular weight standards (low range 10 000–100 000; Bio-Rad) were used as markers. Electrophoresis was carried out in 25 mM Tris–glycine buffer (pH 8.3) containing 0.1% SDS at 30 mA. Stain and destain of gels were carried out as above described.

Isoelectrofocusing (IEF) and Two-Dimensional Electrophoresis (2-DE). Proteins were separated in the first dimension by IEF according to the method described by Osterman (1984) in a polyacrylamide gel containing 2% preblended ampholine (pH 5.0–8.0) (LKB) on an LKB 2117 Multiphor apparatus. Protein samples were dissolved in 1% glycine, and IEF was carried out at 25 W for 2.5 h. Thereafter, the proteins were separated in the second dimension by molecular weight according to O'Farrel (1975), and the gel was stained with Silver Stain (Bio-Rad). For

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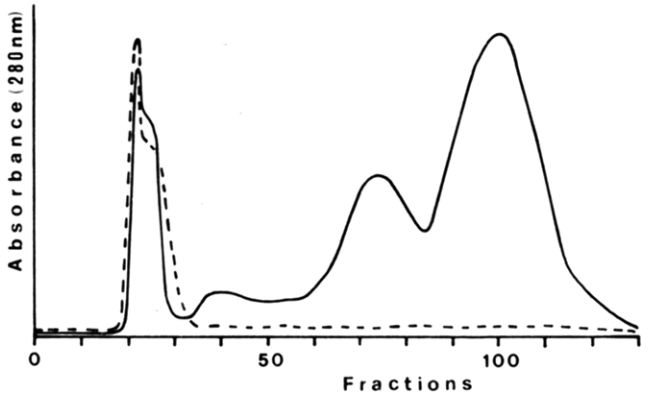


Figure 1. Sephadex G-50 fractionation of sunflower globulin extracted at alkaline pH (continuous line) and at acidic pH (dashed line).

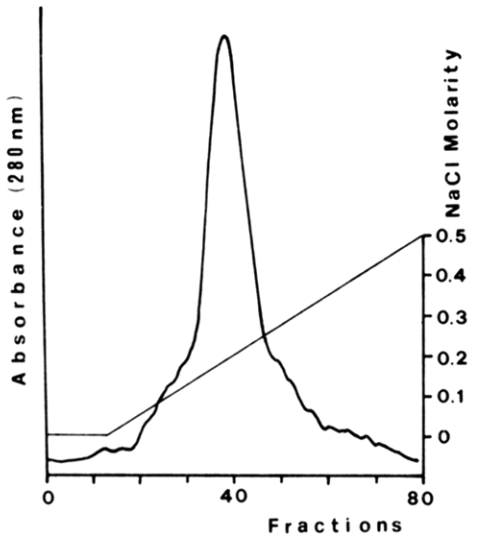


Figure 2. DEAE-cellulose chromatographic pattern of helianthinin extracted at acid pH.

determinations of p*I* values, the Sigma IEF-Mixer 3.5–9.3 markers were utilized.

RESULTS AND DISCUSSION

Different problems arose during the isolation and purification step of the major protein fraction of sunflower seeds. Extractions in alkaline conditions according to Baudet and Mossé (1977) showed contamination with low molecular weight albumin fractions and the presence of bound chlorogenic acid that can be removed after gel chromatography. As shown in Figure 1, proteins elute with the void volume while a second peak containing nucleic acids and a third fraction with low molecular weights show an absorbance range typical of chlorogenic acid. In order to avoid such contamination and to obtain a more rapid isolation procedure without the need for gel chromatography fractionation, we extracted the globulin fraction at acid pH as described under Materials and Methods. This procedure allows the removal of albumins and of chlorogenic acid at the same time in the supernatants of the first centrifugations. Globulins are recovered after solubilization of the pellet at alkaline pH. The chromatography profile of these proteins is shown in Figure 1. The elution profile after ion-exchange chromatography indicates the presence of a single symmetrical peak (Figure 2) eluting with 0.2 M NaCl. Globulins extracted with different methods have been compared by electrophoretic analyses. In Figure 3 are reported the electrophoretic patterns of the proteins extracted at acid pH and from protein bodies in

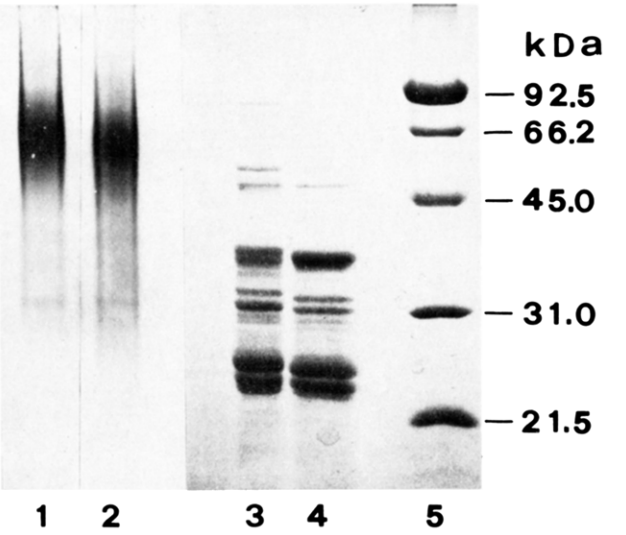
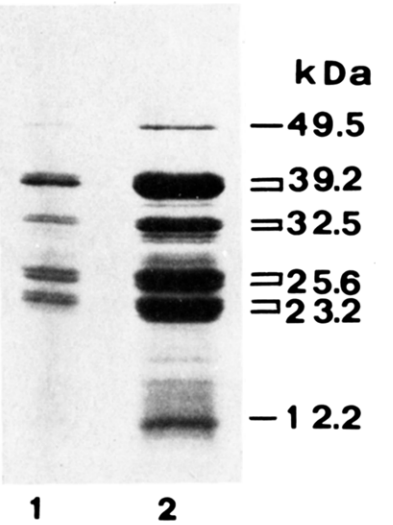


Figure 3. Electrophoresis of helianthinin extracted at acidic pH (lanes 1 and 3) and from protein bodies (lanes 2 and 4) under normal (lanes 1 and 2) and denaturing (lanes 3 and 4) conditions. Lane 5 represents protein markers for SDS-PAGE.



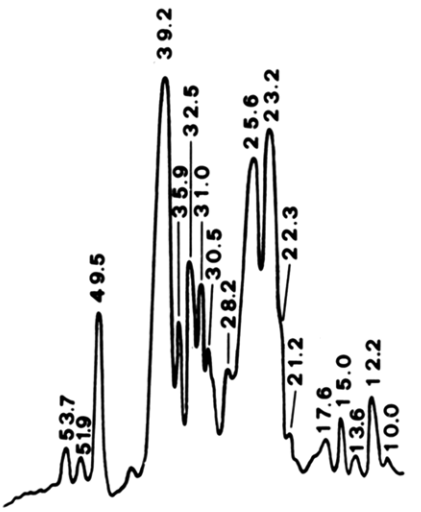


Figure 4. Helianthinin SDS-PAGE: (top) electrophoretic patterns with 40 μg (lane 1) and 100 μg (lane 2) of protein; (bottom) densitometric tracing.

normal and denaturing conditions. After PAGE it is possible to see a wide band with the same mobility in the material extracted at pH 5.0 and from the protein bodies

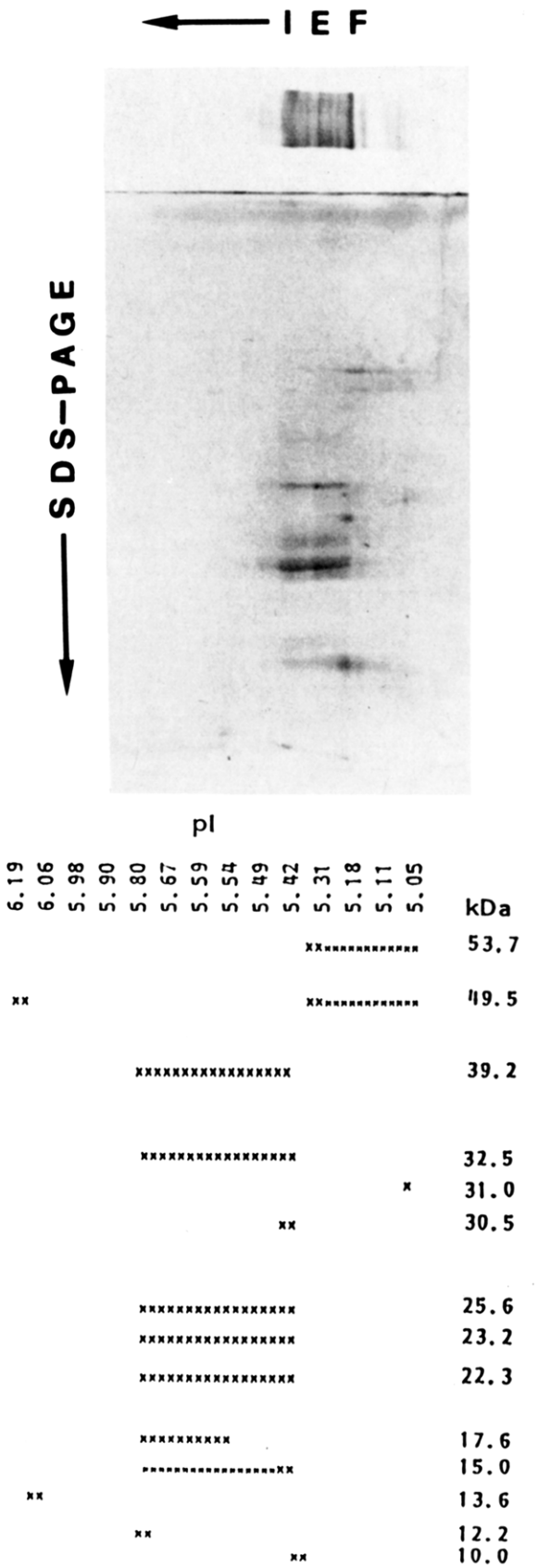


Figure 5. (Top) Two-dimensional electrophoresis (IEF/SDS-PAGE) of helianthinin. (Bottom) Schematic drawing.

(molecular weight 300K). Similarly, the SDS-PAGE patterns of the globulins extracted with the two methods are identical. As better evidenced in Figure 4, the SDS-PAGE shows the presence of four major bands with molecular weights 39.2, 32.5, 25.6, and 23.2K. As a matter of fact, each band is composed of two polypeptides. Some minor components are present with molecular weights ranging from 49.5 to 53.7K, from 10.0 to 21.2K, and others. The molecular weight values of each component can be more clearly evaluated from the electrophoretic pattern and from the corresponding densitometric tracings of Figure 4.

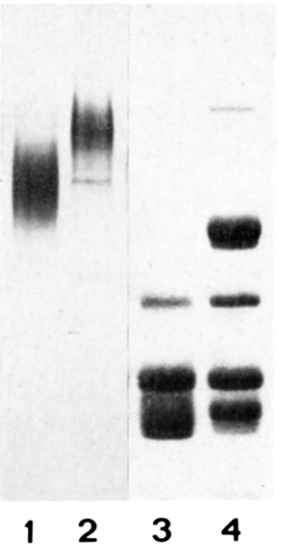


Figure 6. PAGE (lanes 1 and 2) and SDS-PAGE (lanes 3 and 4) of globulin extracted from two sublines of the M211 sunflower line.

Charge heterogeneity of the polypeptides was estimated by two-dimensional electrophoresis using isoelectrofocusing in the first dimension and SDS-PAGE in the second dimension. Helianthinin subunits show charge heterogeneities in a p*I* range of 5.0–6.2 (Figure 5 (top)); the correspondence between molecular weight of the subunits and p*I* is better evidenced in the schematic arrangement of Figure 5 (bottom).

The electrophoretic patterns under denaturating conditions are comparable with those obtained by Allen et al. (1985). The 50K bands are probably precursor molecules, as indicated by our preliminary results on protein labeling at different stages of seed maturation.

It is worth noting that our extraction method allows the purification of helianthinin from seed fragments in a short time, thus enabling the simultaneous preparation of several samples. In this way it is possible to screen several seeds coming from different populations or lines with a nondestructive method and to recover the seeds with particular electrophoretic patterns for sowing and propagation. We have shown that different selfed lines can be characterized by their electrophoretic patterns of seed storage globulins. PAGE analyses of single-seed-extracted globulin have shown that native helianthinin can alternatively have an apparent molecular weight of 300K or of 190K; in some cases a 440K band was found. Our results agree well with those obtained by Dalgalarrondo et al. (1984). Interestingly enough, the subunit composition of the 300K and 190K fractions is different as judged from SDS-PAGE; in Figure 6 are reported the electrophoretic patterns of the globulins extracted from the line M221 obtained in our laboratory. In this line, two different sublines have been isolated, one of which has the 300K fraction and the normal subunit pattern, while the other has the 190K globulin and an SDS-PAGE pattern lacking the high molecular weight components (39.2K). Our analyses of several other lines (manuscript in preparation) have shown that the electrophoretic pattern of the helianthinin subunits is a peculiar

feature of each line. Work is in progress to ascertain whether these differences are linked to alternative pathways of maturation of the precursor molecules.

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LITERATURE CITED

Allen, R. D.; Nessler, C. L.; Thomas, T. L. Developmental Expression of Sunflower 11S Storage Protein Genes. *Plant Mol. Biol.* **1985**, *5*, 165–173.

Baudet, J.; Mossé, J. Fractionation of Sunflower Seed Proteins. *J. Am. Oil Chem. Soc.* **1977**, *54*, 82A–86A.

Buttrose, M. S.; Lott, J. N. A. Inclusions in Seed Protein Bodies in Members of the Compositae and Anacardiaceae: Comparison with Other Dicotyledonous Families. *Can. J. Bot.* **1977**, *56*, 2062–2071.

Dalgalarrondo, M.; Raymond, J.; Azanza, J. L. Sunflower Seed Proteins: Characterization and Subunit Composition of the Globulin Fraction. *J. Exp. Bot.* **1984**, *35*, 1618–1628.

Dalgalarrondo, M.; Raymond, J.; Azanza, J. L. Sunflower Seed Protein: Size and Charge Heterogeneity in Subunits of the Globulin Fraction. *Biochim.* **1985**, *67*, 629–632.

Davis, B. J. Disc Electrophoresis-II Method and Application to Human Serum Proteins. *Ann. N.Y Acad. Sci.* **1964**, *121*, 404–427.

Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head Bacteriophage T4. *Nature* **1970**, *227*, 680–689.

O'Farrel, P. H. High Resolution Two-dimensional Electrophoresis of Proteins. *J. Biol. Chem.* **1975**, *250*, 4007–4021.

Osterman, L. A. Isoelectric Focusing (IEF). In *Methods of Protein and Nucleic Acid Research*; Springer-Verlag: Berlin, Heidelberg, 1984.

Pusztai, A.; Croy, R. R. D.; Grant, G.; Watt, W. B. Compartimentalization in the Cotyledonary Cells of *Phaseolus vulgaris* L. Seeds: a Differential Sedimentation Study. *New Phytol.* **1977**, *79*, 61–71.

Reichelt, R.; Schwenke, K. D.; Konig T.; Pahtz, W.; Wangermann, G. Electron Microscopic Studies for Estimation of the Quaternary Structure of the 11S Globulin (Helianthinin) from Sunflower Seed (*Helianthus annuus L.*). *Biochem. Physiol. Pflanz.* **1980**, *125*, 653–663.

Sabir, M. A.; Sosulski, F. W.; Mackenzie, S. L. Gel Chromatography of Sunflower Proteins. *J. Agric. Food Chem.* **1973**, *21*, 988–993.

Schwenke, K. D.; Schultz, M.; Linow, K. J.; Uhlig, J.; Franzke, C. L. Ueber Samenproteine. 4. Mitt. Isolierung der Globulin-Hauptkomponente aus Sonnenblumensamen. *Nahrung* **1974**, *18*, 709–719.

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Pattern of Zinc-65 Incorporation into Soybean Seeds by Root Absorption, Stem Injection, and Foliar Application[1]

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The pattern of ^{65}Zn incorporation into soybean seeds of plants grown hydroponically and intrinsically labeled with ^{65}Zn by root absorption, stem injection, and foliar application was studied. Stem injection resulted in the greatest (64.5% of dose) accumulation of ^{65}Zn while incorporation of ^{65}Zn through root absorption was the least (23.4%) and through foliar application was intermediate (37.5%). Regardless of the labeling techniques, approximately 40–45% of the seed ^{65}Zn was associated with the subcellular organelles. The pattern of zinc incorporation did not change appreciably as a result of the labeling technique. The major portion of the soluble zinc was not associated with the major proteins (11S and 7S) of soybeans but either was free or was associated with very low molecular weight amino acids, peptides, or their complexes with phytic acid. Zinc in soybean seems to be ionically bound, and this association is affected by the pH of the extracting buffer.

The bioavailability and chemical association of zinc in soybeans can be studied with radiotracers. Incorporation of ^{65}Zn into soybeans can be accomplished either by extrinsic labeling where the radionuclide is physically mixed with soy or by intrinsic labeling where ^{65}Zn is incorporated into the plant system biologically (Weaver, 1985). If extrinsic labeling of ^{65}Zn exchanges completely with the endogenous zinc, then extrinsic labeling would be an easy approach as it is less costly and easier than intrinsic labeling (Evans and Johnson, 1977). However, individual foods and processing conditions should be checked before assuming that extrinsic labels exchange with endogenous zinc (Weaver, 1984, 1985).

Intrinsic labeling is assumed to incorporate ^{65}Zn into the plant in the same pattern as zinc is incorporated in the general field conditions. Although intrinsic labeling could be done through soil media, it is not practical because of the low efficiency of incorporation of an applied dose (Weaver, 1984, 1985). Labeling through hydroponic culture (root absorption) is more practical and efficient in comparison to soil (Weaver, 1985). However, labeling through hydroponic culture (root absorption) is less efficient in terms of incorporation and more specialized, laborious, and time-consuming than stem injection and foliar application (Weaver, 1984, 1985; Schmitt and Weaver, 1984; Janghorbani et al., 1983; Starks and Johnson, 1985; Zeind, 1976). Stem injection usually incorporates a higher percentage of the applied dose into the seeds than root ab-

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