Isolation and Purification of Cottonseed 7S Storage Protein and Its Subunits

Henry F. Marshall, Jr.

U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, P.O. Box 19687, New Orleans, Louisiana 70179

A rapid, simple method was developed for isolating and purifying the 7S storage globulin of cottonseed and its two major subunits. By use of small amounts of starting material, extractions were performed at 4 °C except when otherwise necessary, preparation times are kept to a minimum, and, most importantly, purification of the storage protein and its subunits is accomplished with high-performance liquid chromatography. A reverse-phase chromatography method was used to isolate and purify the subunits in one step. The 7S globulin was determined to have a molecular weight of 98K and has two subunits of 54K and 48K. There was no covalent linkage between the subunits. Results indicate that the 7S storage protein has a simpler subunit composition than previously reported.

INTRODUCTION

Cottonseed, due to its high protein content, has been the subject of many investigations as a potential food source for human consumption. The principal determinants are its good nutritional quality and functional properties (Elmore and King, 1978; Castro et al., 1976; Martinez et al., 1970).

The storage globulins make up approximately 70% of the protein present in cottonseed, principally the 11S and 7S components with the latter having been the focus of most studies for physical characterization. Procedures for the isolation and purification of these two globulin components have been well described in the literature (Rossi-Fanelli et al., 1964; Wallace, 1976; Zarins and Cherry, 1981; Dure and Chlan, 1981; Zarins et al., 1984; Reddy and Rao, 1988a). Methods for obtaining the principal subunits of the globulins have been reported by Wallace (1976) and King (1980). For these procedures, relatively large amounts of defatted cottonseed flour or meal are used in batch isolation procedures which take advantage of the solubility characteristics of the storage globulins. Subsequent purifications are then accomplished by traditional liquid chromatography using gel filtration and/or ion-exchange methods. Lengthy periods of time for these approaches are unavoidable and may lead to altered proteins unsuitable for structural studies, or proteolytic degradation may occur. Additionally, bacteriostatic reagents that are sometimes used may not be totally removed by subsequent dialysis.

The procedure presented here incorporates minimal exposure to ambient temperatures, short handling times, and purification by HPLC. Small amounts of high-purity 7S globulin and its subunits are obtained.

EXPERIMENTAL PROCEDURES

Materials. Glandless cottonseed, cultivar Acala 8160, was used for the protein extractions. It was stored in the freezer at −10 °C until used.

Reagents for the LDS–PAGE electrophoresis were obtained from Sigma Chemical Co., St. Louis, MO. The silver stain kit used for the staining of the PAGE gels was obtained from the Chemical Division of Bio-Rad, Richmond CA. Sodium chloride, mono- and dibasic potassium phosphates, and the solvents for fat extraction were of reagent grade from J. T. Baker Chemical Co. The Superose 12 FPLC column was obtained from Pharmacia LKB Biotechnology Inc., Piscataway, NJ.

Amino acid analysis elution buffers formulated for the Beckman System 6300 amino acid analyzer were from Beckman Instruments, Spinco Division, Palo Alto, CA.

Methods. *Preparation of Defatted Meal.* Cottonseed kernels were ground with dry ice in a Tekmar mill. The dry ice was allowed to evaporate at ambient temperature. The full fat meal obtained from this procedure was extracted (3×) at ambient temperature with a 3:1 chloroform–methanol mixture. Next, the meal was extracted (3×) with MeOH–H_2O (80:20) at 4 °C. Finally, the meal was extracted once with diethyl ether at 4 °C. For these extractions a 10:1 ratio of solvent to meal was used.

Protein Extraction and Isolation. The 7S globulin was extracted from defatted meal by a modified procedure described by Wallace (1976). A flow chart of the procedure is presented in Figure 1. The starting amount for the extraction was reduced to 1 g. An Omni-Mixer was used for all extractions. The 30 °C extraction for the 7S globulin was performed in a temperature-controlled H_2O bath. All centrifugations were performed with a Eppendorf microfuge, Model 5414, for 3 min at 12000g. This short spin time did not appreciably alter the temperatures required for the different steps of the preparation procedure. Once the 7S globulin precipitate was obtained (Figure 1), it could be resolubilized in 10% NaCl and directly purified with the Superose 12 column or frozen and stored until needed. For most situations it was directly purified in the following manner.

Protein Purification. A Superose 12 column with 10% NaCl, pH 7.0, as the eluant was used for the purification of the 7S globulin. Protein concentration was adjusted to 33.0 mg/mL, and 100-μL aliquots were applied to the column for each run. The flow rate was 0.5 mL/min, and the eluant was monitored at 280 nm. Fractions were collected with a Pharmacia Frac-100 fraction collector set at a 45% threshold value. After purification, the collected fractions were pooled and the material was exhaustively dialyzed against H_2O at 4 °C and then lyophilized. The HPLC apparatus used included a Kratos Spectra-Flow 430 gradient former, a Beckman 112 pump, a Kratos SF 770R variable-wavelength detector, and a Hewlett-Packard 3390A integrator.

Subunit Isolation. Isolation and purification of the individual subunits was accomplished in one step with a Bio-Rad RP-318 Hi-Pore reversed-phase column. For this step, protein concentration was 3.0 mg/mL 0.1% trifluoroacetic acid (TFA), with 100-μL aliquots injected. The column flow rate was 1.0 mL/min. The column was preequilibrated with 30% acetonitrile–0.1% TFA prior to sample application. The gradient for

This article not subject to U.S. Copyright. Published 1990 by the American Chemical Society

PREPARATION PROCEDURE FOR 7S GLOBULIN

Defatted Cottonseed Meal
Extract 2X (1:15::w/v) with H_2O, 4C
30 minutes
Centrifuge

Precipitate
Extract 1X (1:15::w/v) with 10% NaCl, pH 7.0
4C, 30 minutes
Centrifuge

Supernatant
Storage Protein Fraction
Dialyze against H_2O (4X), 4C
Centrifuge

Precipitate
Extract 1X (1:15::w/v) with 0.3M NaCl, pH 7.0
30C, 30 minutes
Centrifuge

Supernatant
Cryoprecipitate, 4C
Centrifuge

Precipitate
7S Globulin
Dissolve in 10% NaCl, pH 7.0
Freeze precipitate
or
Purify directly with Superose 12 column

Figure 1. Isolation procedure for preparation of cottonseed 7S globulin.

the reverse-phase separation was from 30% to 45% acetonitrile–0.1% TFA for 30 min and then a hold at 45% acetonitrile–0.1% TFA for 10 min. After this period of time, the column was washed with 100% acetonitrile–0.1% TFA for 1 min and then reequilibrated to the starting conditions. The effluent was monitored at 214 nm. The threshold value for the Frac-100 fraction collector was set at 45% full scale for these purifications.

LDS–PAGE Electrophoresis. Polyacrylamide gel electrophoresis was performed under denaturing and reducing conditions. A modification of the method of Laemmli (1970) was used. Lithium dodecyl sulfate (LDS) was used in lieu of SDS. The lithium salt has a higher solubility at lowered temperatures and behaves in identical fashion with SDS in polyacrylamide gels (Piccioni et al., 1982). For these experiments gels were run at ambient temperatures. Sample amounts were 30 μg/10 μL per well, and the gels were silver stained by using a Bio-Rad silver stain kit.

Amino Acid Analysis. Protein hydrolysates for amino acid analysis were prepared by the method of Moore and Stein (1963). Amino acid analyses were performed on a Beckman System 6300 amino acid analyzer. Residues of amino acids per mole for each purified protein were determined from the molar ratios for each amino acid with the mole ratio of arginine set to 1. Molecular weights for each protein were estimated from HPLC gel filtration data obtained with the use of standard proteins.

RESULTS AND DISCUSSION

Isolation of the 7S Globulin. Cottonseed storage proteins will have a stable native structure a pH 7.0. The 7S globulin will dimerize in the presence of phosphate ions, but using only NaCl solutions at pH 7.0 precluded this possibility (Wallace, 1976). Temperatures maintained at 4 °C should reduce possible proteolysis.

After extraction of the albumin fraction with cold H_2O, the storage proteins, principally the 7S component, were

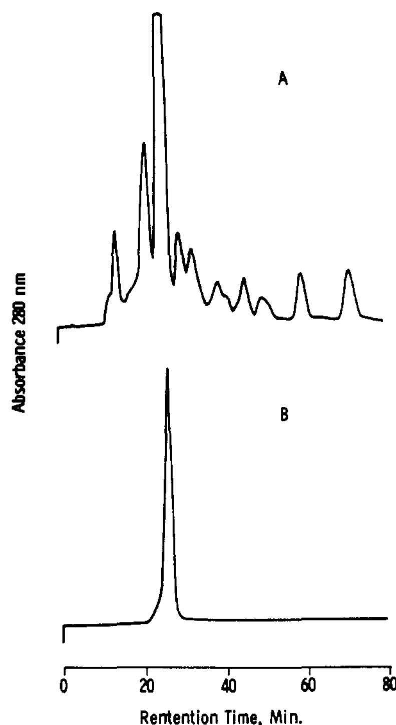

Figure 2. (A) Chromatography of storage protein fraction. Gel filtration was performed by using a Superose 12 column; 10% NaCl, pH 7.0, is the eluant. (B) Chromatography of cryoprecipiate from purification scheme, Figure 1. Gel filtration was performed by using a superose 12 column; 10% NaCl, pH 7.0, is the eluant.

extracted at 4 °C with 10% NaCl. The 4 °C temperature is advantageous for 7S isolation as the 11S globulin displays a lowered solubility at this temperature. Gel filtration of the 10% NaCl fraction (Figure 2A) shows the 7S globulin to be the preponderant species present.

The 7S globulin was obtained in high purity by utilizing its reduced solubility in 0.3 M NaCl at 30 °C. Figure 2B illustrates this fact quite dramatically. Prior to chromatography with the Superose 12 column, the protein obtained from the cryoprecipitation consistently averaged at least 95% purity (as determined by LDS electrophoresis). For the chromatography of the 7S globulin, the fraction collector was programmed to collect fractions only after reaching a 45% threshold value. When the fraction collection was begun at this point, the globulin was obtained at >99% purity. The purified globulin was analyzed by LDS electrophoresis (Figure 3). The results show that the 7S globulin consists of only two large subunits having molecular weights of 54K and 48K. This subunit composition differs from that in earlier papers (Wallace, 1976; Dieckert et al., 1981; Zarins and Cherry, 1981; Marshall et al., 1984; Zarins et al., 1984; Reddy and Rao, 1988b) because of the conspicuous absence of smaller molecular weight components.

Reverse-phase chromatography of the 7S globulin with a 30–45% acetonitrile–0.1% TFA gradient resulted in two well-resolved major peaks (Figure 4). Again, collection of the two fractions was delayed until a 45% threshold value was reached on the fraction collector. LDS electrophoresis profiles (Figure 3) indicate each fraction to be homogeneous single polypeptides. The subunits have been designated I (54K) and II (48K).

Since the separation of the two subunits was accomplished with reverse-phase chromatography and reverse-phase chromatography separates on the basis of hydrophobic interactions only, it is clear that the subunits are not covalently linked.

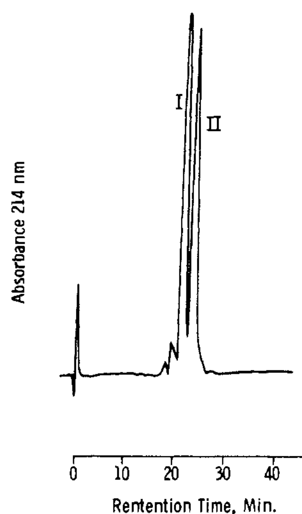

Figure 3. Electrophoretic profiles for 7S, subunits I and II. Gels were silver stained. Concentrations were 30 μg/10 μL per well for each sample and 2 μg/10 mL per well for the standard.

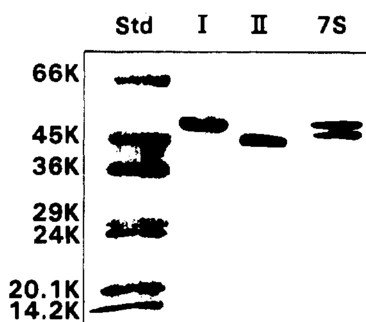

Figure 4. Reverse-phase chromatography of purified 7S cottonseed globulin on a Bio-Rad RP-328 Hi-Pore column with a linear gradient of 30–45% acetonitrile–0.1% TFA for 30 min, 45% acetonitrile–0.1% TFA for 10 min, then to 100% acetonitrile–0.1% TFA, 1.0 min.

King (1980) has performed isoelectric focusing on the subunits of the 7S globulin and demonstrated the presence of at least 9 visualized components for the 54K subunit and 12 for the 48K subunit covering the pH range 6.7–8.3. No isoelectric focusing was performed on the subunits obtained in these experiments; however, in determining an optimal gradient for the subunit separation, it was noticed that a less sharp gradient would give rise to more than two peaks, suggesting that perhaps some of the genetic variants of the subunits may be separable by reverse-phase chromatography or hydrophobic interaction chromatography.

The amino acid profiles were determined for the 7S globulin and for subunits I and II (Table I). The molecular weights determined from the amino acid composition for the two subunits are in good agreement with those determined from the electrophoresis. The profile for the 48K subunit is quite similar to that reported by Dure and Chlan (1981). Interestingly, the molecular weight for the 7S globulin as determined by amino acid analysis is considerably less than previously reported (Wallace, 1976; Zarins and Cherry, 1981; Reddy and Rao, 1988). On the basis of our electrophoretic 7S profile this is not surprising since the results indicate only two components present, while other papers show a more complex composition of seven or eight components (Wallace, 1976; Dieckert et al., 1981; Zarins and Cherry, 1981; Marshall et al., 1981; Zarins et al., 1984; Reddy and Rao, 1988). Amino acid analysis of the 7S globulin does substantiate evidence that the 7S globulin is composed of only two subunits since the total

Table I. Amino Acid Composition of 7S Storage Protein, Subunit I (54K), and Subunit II (48K)

amino acid	7S res/MW 99 035	subunit I res/MW 53 500	subunit II res/MW 48 100
Asx	85	44	43
Thr	29	16	16
Ser	58	33	28
Glx	165	94	78
Pro	40	24	18
Gly	58	28	34
Ala	46	25	23
$^1/_2$ Cys	nd[a]	nd	nd
Val	57	29	31
Met	nd	nd	nd
Ile	29	17	13
Leu	56	31	26
Tyr	24	12	12
Phe	62	34	29
Lys	26	12	14
His	27	14	14
Arg	87	48	41
Trp	nd	nd	nd

[a] nd, not determined.

amino acid composition and molecular weight exhibit an almost stoichiometric relationship to the sum of 54K and 48K subunits.

For the isolation and purification of the 7S globulin, preparation times were kept to a minimum, and temperatures were maintained at 4 °C except where otherwise necessary. Purification was accomplished with HPLC chromatography, which is superior to standard liquid chromatography in its ability to separate mixtures of components. Additionally, rather severe cuts were imposed during the collection of the purified fractions as dictated by the high threshold value setting on the fraction collector. The combined effects of this approach resulted in fractions of extremely high purity. Previously reported smaller molecular weight components in the 7S globulin may perhaps be carry-over contaminants from the isolation and/or proteolytic digestion products which were not able to be separated from the 7S globulin by conventional liquid chromatographic methods.

The electrophoretic profiles indicated that with reverse-phase chromatography and the proper gradient the subunits of the 7S cottonseed globulin can be obtained in high purity in one step. Milligram quantities of highly purified subunits are possible in a relatively short amount of time. No dialysis of the purified fractions is necessary since the fractions can be directly lyophilized since only acetonitrile, TFA, and H_2O are present.

Analytical size columns were used for the separations and purifications; however, they performed quite efficiently, and since retention times were low, 16–20 separations per day can be made.

Purified subunits can be obtained from cottonseed kernel in 2–2.5 days. The method is applicable where large numbers of varieties of seed are being compared or where small amounts of highly purified protein or subunits are required. This approach should be applicable to other oilseed protein systems.

ACKNOWLEDGMENT

I thank Lisa C. Bowers for her excellent assistance.

LITERATURE CITED

Castro, C. E.; Yang, S. P.; Harden, M. L. Supplemental Value of Liquid Cyclone Processed Cottonseed Flour on the Proteins of Soybean Products and Cereals. *Cereal Chem.* **1976**, *53*, 291–298.

Dieckert, J. W.; Wallace, R. W.; Dieckert, M. W. Chemistry and Biology of the Cottonseed Globulins. *Proc.-Cotton Dust Res. Conf., Beltwide Cotton Prod. Res. Conf.* **1981**, 351–355.

Dure, L., III; Chlan, C. Developmental Biochemistry of Cottonseed Embryogensis and Germination. XII. Purification and Properties of Principal Storage Proteins. *Plant Physiol.* **1981**, *68*, 180–186.

Elmore, C. D.; King, E. E. Amino Acid Composition of Germinating Cotton Seeds. *Plant Physiol.* **1978**, *62*, 531–535.

King, E. E. Compositional Relationships Among Electrophoretic Isolates From Cottonseed Bodies. *Phytochemistry* **1980**, *19*, 1647–1651.

Laemmli, U. K. Cleavage of Structural Protein During the Assembly of the Head of Bacteriophage T4. *Nature* **1979**, *227*, 680–685.

Marshall, H. F.; Shirer, M. A.; Cherry, J. P. Characterization of Glandless Cottonseed Storage Proteins by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. *Cereal Chem.* **1984**, *61* (2), 166–168.

Martinez, W. H.; Berardi, L. C.; Glodblatt, L. A. Cottonseed Protein Products—Composition and Functionality. *J. Agric. Food Chem.* **1970**, *18*, 961–968.

Moore, S.; Stein, W. H. Chromatographic Determination of Amino Acids by the Use of Automatic Recording Equipment. *Methods Enzymol.* **1963**, *6*, 819–831.

Piccioni, R.; Bellemare, G.; Chua, N.-H. Methods of Polyacrylamide Gel Electrophoresis in the Analysis and Preparation of Plant Polypeptides. In *Methods in Chloroplast Biology*; Edleman, M., Hallick, R. B., Chua, N.-H., Eds.; Elsevier Biomedical Press: Amsterdam, 1982; pp 985–1014.

Reddy, M. I.; Narasinga Rao, M. S. Method for the Isolation of Gossypin (11S Protein) and Congossypin (7S Protein) from Glanded Cottonseed. *J. Agric. Food Chem.* **1988a**, *36*, 237–240.

Reddy, M. I.; Narasinga Rao, M. S. Physicochemical Properties of Gossypin (11S Protein) and Congossypin (7S Protein) of Glanded Cottonseed. *J. Agric. Food Chem.* **1988b**, *36*, 241–245.

Rossi-Fanelli, A.; Antonini, E.; Brunori, M.; Bruzzesi, M. R.; Caputo, A.; Satriani, F. Isolation of a Monodisperse Protein Fraction From Cottonseed. *Biochem. Biophys. Res. Commun.* **1964**, *15* (2), 110–115.

Wallace, R. W. Isolation and Partial Characterization of the Acalin A and B Fractions of Cottonseed Globulins. Ph.D. Dissertation, Texas A&M University, 1976.

Zarins, Z. M.; Cherry, J. P. Storage Proteins of Glandless Cottonseed Flour. *J. Food Sci.* **1981**, *46*, 1855–1859, 1862.

Zarins, Z. M.; Phillips, R. D.; Martinez, W. H. A Rapid Procedure for Isolation of the 7S Storage Protein of Cottonseed and Its Characterization. *Cereal Chem.* **1984**, *61* (6), 471–474.

Received for review October 2, 1989. Revised manuscript received March 6, 1990. Accepted March 25, 1990.