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Method for the Isolation of Gossypin (11S Protein) and Congossypin (7S Protein) from Glanded Cottonseed

I. Mohan Reddy[1] and M. S. Narasinga Rao*

A method for the isolation of gossypin and congossypin from glanded cottonseed has been developed on the basis of their solubility characteristics as a function of ionic strength and temperature. Further purification of the proteins by gel filtration on Sephadex G-200 column yielded homogeneous (~98%) preparations, as judged by sedimentation velocity, gel electrophoresis, and ion-exchange chromatography. Gossypin and congossypin were characterized by sedimentation constants ($S_{20,w}$) of 11.0 and 7.6 and $E^{1\%}_{1cm}$ at 280 nm of 7.6 and 6.0, respectively. The proteins were free from gossypol (free and bound) and nucleic acid impurities.

Cottonseed proteins consist of three protein fractions designated as 12S, 7S, and 2S on the basis of their sedimentation coefficients (Martinez et al., 1970). The 12S and 7S proteins are the storage proteins and constitute the major fraction of cottonseed proteins (Martinez, 1979). An understanding of the individual protein fractions is essential for their better utilization (King, 1980). Several methods for the isolation of 7S and 12S proteins have been described (Rossi-Fanelli et al., 1964; Ibragimov et al., 1969; Yuldasheva et al., 1975; Youle and Huang, 1979; Zarins and Cherry, 1981; Mohan Reddy et al., 1982; Zarins et al., 1984). Many of the existing procedures for isolating 7S and 12S proteins are tedious, time-consuming, and suitable only for analytical purposes. Further, these methods subject the proteins to extreme changes in environment including pH. It is not certain whether the proteins isolated by these methods are free from gossypol and nucleic acid impurities. Therefore, in this investigation, a method for the isolation of 12S and 7S proteins of cottonseed on a preparative scale is developed and reported here. In analogy with soybean and groundnut proteins (Altschul et al., 1966), we recently suggested the names gossypin and congossypin for the 12S (11S) and 7S protein, respectively, on the basis of the botanical name of cottonseed, *Gossypium herbaceum* (Mohan Reddy, 1985). Recently, Prakash and Narasinga Rao (1986) also suggested names for protein fractions of other oilseeds on the basis of their botanical names. In this paper we use the names gossypin and congossypin.

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570 013, India.
[1]Present address: Institute of Food Science, Cornell University, Ithaca, NY 14853.

EXPERIMENTAL SECTION

Materials. Glanded cottonseeds (*G. herbaceum* var. Jaydhar) were obtained from Karnataka State Seeds Corp. Ltd., Mysore, India, and stored cold.

The source of chemicals used were as follows: Sephadex G-200 (40–120 μm), blue dextran, tris(hydroxymethyl)-aminomethane (Tris), and DNP–lysine were from Sigma Chemical Co.; DEAE-Sephacel was from Pharmacia Fine Chemicals; amido-black and ammonium persulfate were from E. Merck; acrylamide and bis(acrylamide) were from Koch-Light Laboratories; TEMED was from Fluka; bromophenol blue was from BDH.

Methods. *Low-Gossypol Cottonseed Flour.* Defatted cottonseed flakes were prepared by the method described earlier (Mohan Reddy et al., 1982). Low-gossypol cottonseed flour (60 mesh) was prepared according to the method of Damaty and Hudson (1975), by extracting the defatted flakes with 70% aqueous acetone followed by anhydrous acetone. The flour had 0.018% free and 0.07% bound gossypol, compared to 1.50% free and 0.18% bound gossypol in untreated flour (Official AOCS Methods Ba 7-58 and Ba 8-55).

Protein Extraction. Low-gossypol cottonseed flour (20 g) was extracted 3× with 300 mL of water to remove nonstorage proteins and sugars. The storage proteins were then extracted with 200 mL of 0.5 M Tris-HCl buffer of

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pH 7.0 containing 0.5 M NaCl (buffer A) for 1 h and centrifuged. All centrifugations were carried out at 26 °C and 13000*g* for 20 min, unless otherwise stated. Storage protein extract with buffer of ionic strength of 1.0 was used for the isolation of gossypin and congossypin.

Isolation of Gossypin. The Tris extract (200 mL; I = 1.0) was diluted to 520 mL (2.6 × 200 mL; I = 0.385) with distilled water and centrifuged to remove the precipitate. The supernatant (515 mL) was made up to 690 mL so that final I = 0.29 to precipitate the crude gossypin and centrifuged. The supernatant obtained at this stage (~685 mL; I = 0.29) was used for the isolation of congossypin. The precipitate containing crude gossypin was dissolved in buffer A; solid ammonium sulfate (37.5%, w/v) was added to the clear solution with constant stirring, and the solution was kept in cold for 10 min and centrifuged at ~10 °C. the precipitate was discarded, and the protein (gossypin) remaining in the supernatant was concentrated by ammonium sulfate precipitation by raising its concentration to 45% (w/v). The precipitate (gossypin) was dissolved in 0.05 M Tris-HCl buffer, pH 7.0, containing 1.0 M NaCl and 0.02% NaN_3 (buffer B) and centrifuged.

Isolation of Congossypin. Congossypin was isolated by a modification of the procedure of Zarins et al. (1984). The supernatant obtained after the isolation of gossypin (~685 mL; I = 0.29) was further diluted to 1539 mL so that I = 0.13 to precipitate the remaining gossypin and other high molecular weight proteins, and the precipitate was removed by centrifugation. The supernatant was then chilled to 0–2 °C in a freezer, whereupon a precipitate containing fairly homogeneous congossypin was obtained. The mixture was centrifuged in cold and the precipitate dissolved in buffer B and centrifuged.

Purification of Gossypin and Congossypin. The gossypin and congossypin were further purified by gel filtration at room temperature in a 2.6 × 130 cm preparative column of Sephadex G-200 (40–120 μm), using buffer B as eluent. The fractions corresponding to the peak portion were pooled, dialysed thoroughly against distilled water, lyophilized, and stored in an amber-colored bottle in a desiccator, until required. Alternately the precipitate obtained on water dialysis was dissolved in buffer and used.

Gel Filtration. Gel filtration was carried out with Sephadex G-200 (40–120 μm) in buffer B. Preswollen gel was packed into a 2.6 × 130 cm column and equilibrated with the buffer. Sample solution (5.0 mL) containing 100–160 mg of protein was applied to the column and eluted with the same buffer at a constant flow rate of 25 mL/h. The column eluent was collected in 3-mL fractions in an automatic programmable fraction collector and the absorbance at 280 nm monitored.

The void volume (V_0) and total volume (V_t) of the column were determined with use of Blue Dextran and DNP-lysine, respectively.

Ion-Exchange Chromatography. Ion-exchange chromatography was performed with DEAE-Sephacel, in 0.12 M sodium phosphate buffer, pH 8.0. The gel was equilibrated with buffer and packed into a 2.5 × 15 cm column. Approximately 100 mg of protein was loaded and eluted with a continuous linear gradient of 0–0.8 M NaCl in buffer. The gradient was established on a Varigrad apparatus. A constant flow rate of 25 mL/h was maintained, 5-mL fractions were collected in an automatic programmable fraction collector, and absorbance at 280 nm was monitored. NaCl was monitored in the fractions by Mohr's titration (Vogel, 1961).

Polyacrylamide Gel Electrophoresis (PAGE). Gel electrophoresis was carried out in a Shandon vertical gel electrophoresis unit, using 6.5% gels (acrylamide to bis-(acrylamide) ratio 20:1) in 0.12 M sodium phosphate buffer, pH 8.0. Protein samples (60 μg) containing 10% sucrose and 0.05% bromophenol blue (indicator dye) were loaded on the gel, and electrophoresis was performed at a constant current of 4 mA/gel. The gels were stained in 0.5% amido black in 7.5% (v/v) acetic acid and diffusion destained in 7.5% acetic acid.

Sedimentation Velocity. Sedimentation velocity experiments were carried out at 27 °C in a Spinco Model E analytical ultracentrifuge equipped with phase-plate Schlieren optics and rotor temperature indicator and control (RTIC) unit, using a standard 12-mm single-sector Kel F cell centerpiece and 1% protein solution at a speed of 59780 rpm. The plates were read in a Abbe comparator adopted to read ultracentrifuge plates and $s_{20,w}$ values calculated by the standard procedure (Schachman, 1959).

Protein Concentration. This was routinely determined by measuring the absorbance of the protein solution at 280 nm. The absorption coefficient (E) of 1% protein solution in a 1-cm cell at 280 nm, $E^{1\%}_{1cm}$ was determined by plotting absorbance at 280 nm vs protein concentration determined by micro-Kjeldahl nitrogen estimation. A factor of 6.25 was used to convert nitrogen to protein value. The values for gossypin and congossypin were 7.6 and 6.0, respectively, in 1 M NaCl.

Free and Bound Gossypol. Free and bound gossypol content of the proteins was determined by the method of Shandong (1982), using 200 mg of lyophilized protein.

Phosphorus Estimation. An aliquot (1.0 mL) of 2% protein solution in 1 M NaCl was digested successively with 10 N H_2SO_4 and perchloric acid. The phosphorus content of the digested sample was estimated by the method of Taussky and Shorr (1953).

RESULTS AND DISCUSSION

Nomenclature for cottonseed proteins has not been established except for acalin A (7S) and acalin B (12S), isolated from Acala glandless cottonseed (Rossi-Fanelli et al., 1964; Zarins and Cherry, 1981). In the discussion, 11S and 7S proteins of cottonseed will be referred to as gossypin and congossypin, respectively.

Isolation of Gossypin and Congossypin. Low-gossypol cottonseed flour was used in order to eliminate gossypol contamination in isolated protein fractions. Neutral pH and Tris buffer were chosen for the following reasons: (i) The storage proteins are least soluble at pH 7.0 and therefore will have stable native structure at this pH. (ii) Congossypin is known to aggregate in dilute NaCl in the presence of phosphate ions (Zarins and Cherry, 1981); therefore Tris buffer of high molarity (0.5 M) was used to maintain neutral pH during subsequent dilutions.

Gossypin. The gel filtration pattern of storage proteins (Figure 1) consisted of three peaks, designated I–III. They occurred in nearly equal proportions. Peak I corresponds to an aggregate of large molecular weight proteins, while peaks II and III correspond to gossypin and congossypin, respectively (Zarins and Cherry, 1981). The proteins corresponding to peak I were precipitated when the ionic strength of the Tris extract was lowered from 1.0 to 0.385. Selective precipitation of gossypin occurred when the ionic strength was further reduced to 0.29 (Figure 2a). The precipitate consisted of about 73% gossypin and 27% large molecular weight proteins that could be removed by ammonium sulfate (37.5%, w/v) precipitation (Figure 2b). The protein was further purified by gel filtration chromatography on a Sephadex G-200 column (Figure 2c).

Congossypin. Congossypin was isolated by a modification of the procedure on Zarins et al. (1984). It involves

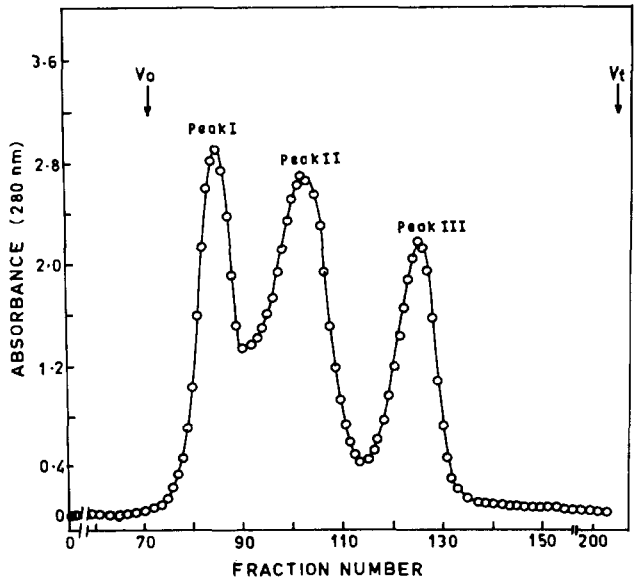

Figure 1. Gel filtration pattern of storage proteins of cottonseed in 0.05 M Tris-HCl buffer of pH 7.0 containing 1.0 M NaCl and 0.02% NaN_3.

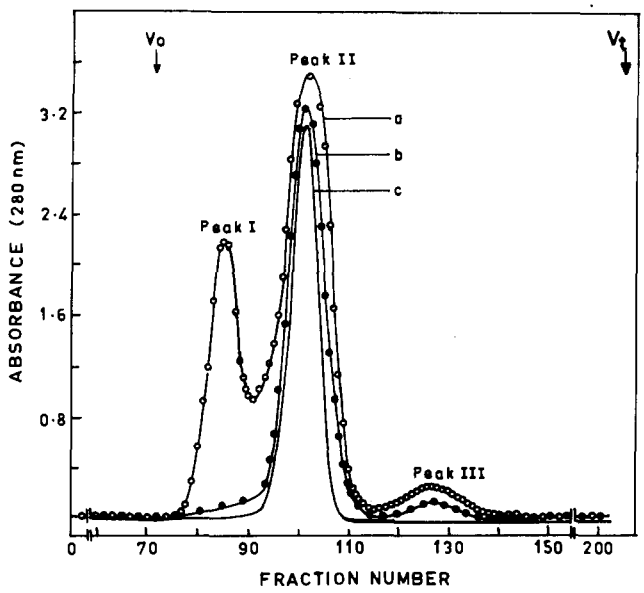

Figure 2. Gel filtration patterns: (a) crude gossypin precipitated at I = 0.23; (b) gossypin precipitated between 37.5 and 45.0% $(NH_4)_2SO_4$; (c) rechromatographed gossypin in 0.05 M Tris-HCl buffer at pH 7.0 containing 1.0 M NaCl and 0.02% NaN_3.

the selective extraction of storage proteins with a solution containing 0.5 M Tris, 0.5 M NaCl, and 0.2 g/L NaN_3 (adjusted to pH 7.0) and 1:3 dilution of this extract with distilled water to precipitate gossypin and large molecular weight proteins and centrifugation. The diluted extract is then chilled to 0 °C to cryoprecipitate congossypin. This precipitate contained about 88% congossypin and 12% gossypin and large molecular weight proteins. The protein was further purified by three-step gel filtration chromatography in a 10 × 90 cm column of Sephadex G-200, using original extracting buffer.

Our modification of the procedure consisted of the following: (i) The supernatant ($I \approx 0.29$) obtained after the precipitation of gossypin was used to isolate congossypin. (ii) This supernatant was diluted to an ionic strength of 0.13, when gossypin and the large molecular weight proteins were completely precipitated. (iii) The congossypin was cryoprecipitated by cooling the supernatant to 0 °C (Figure 3a). (iv) The protein was further

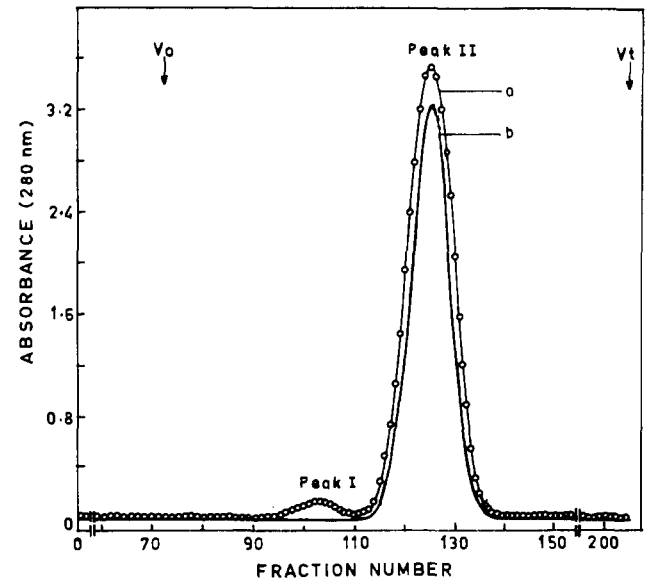

Figure 3. Gel filtration patterns: (a) cold precipitated (0.2 °C) congossypin; (b) rechromatographed congossypin in 0.05 M Tris-HCl buffer of pH 7.0 containing 1.0 M NaCl and 0.02% NaN_3.

purified by a single-step gel filtration chromatography in a 2.6 × 130 cm column of Sephadex G-200 using 0.05 M Tris-HCl buffer of pH 7.0 containing 1.0 M NaCl and 0.02% NaN_3 (Figure 3b). Increasing the length of the column avoided repeated chromatography to obtain a pure protein.

From 100 g of defatted cottonseed flour containing ~50 g of protein, 2.5 g of gossypin and 2.0 g of congossypin can be obtained by this procedure.

Homogeneity of Gossypin and Congossypin. The homogeneity of the proteins was determined by gel filtration ion-exchange chromatography, gel electrophoresis, and sedimentation velocity. The gel filtration pattern of gossypin and congossypin consisted of a single symmetrical peak eluting at V_e/V_o of 1.40 and 1.74, respectively (Figures 2c and 3b). These values coincided with those of peak II and III, respectively, of cottonseed storage proteins (Figure 1).

The DEAE-Sephacel chromatography pattern of gossypin and congossypin consisted of a single symmetrical peak eluting at a salt concentration of 0.185 and 0.205 M NaCl, respectively (Figure 4A,B).

Gel electrophoresis patterns of the proteins showed a single sharp band (Figure 5A,B). Gossypin had low mobility compared to congossypin, which was fairly high.

In sedimentation velocity, gossypin gave a symmetrical major peak with $S_{20,w}$ values of 11.0 S and a small faster moving component of 15.6 S, which formed about 2% of the total (Figure 6A). Congossypin gave a symmetrical major peak with an $S_{20,w}$ value of 7.6 S and a faster moving component of 11.0 S, which too formed about 2% of the total (Figure 6B).

Gel filtration, ion-exchange chromatography, and gel electrophoresis did not show the presence of any impurities in the isolated protein fractions. Only sedimentation velocity technique showed the presence of a high molecular weight impurity, which, however, did not constitute more than 2%.

The extinction coefficient, $E^{1\%}_{1cm}$ at 280 nm, of gossypin was 7.6 and that of congossypin was 6.0, in 1 M NaCl. These values were used to determine the concentration of gossypin and congossypin in solution. A value of $E^{1\%}_{1cm}$ at 280 nm of 6.0 in 1 M NaCl (Mohan Reddy et al., 1982) and 5.95 in 0.5 M Tris–0.5 M NaCl, pH 7.0 (Zarins et al., 1984),

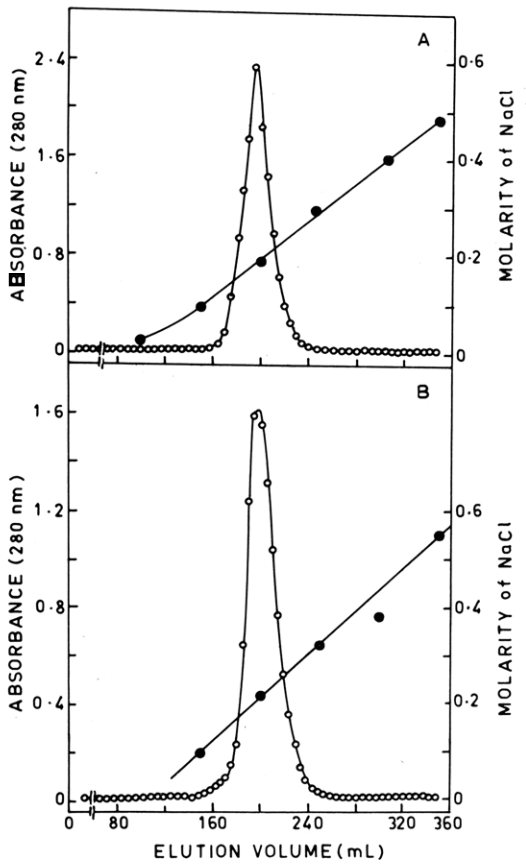

Figure 4. DEAE-Sephacel chromatography pattern of (A) gossypin and (B) congossypin in 0.12 M phosphate buffer, pH 8.0.

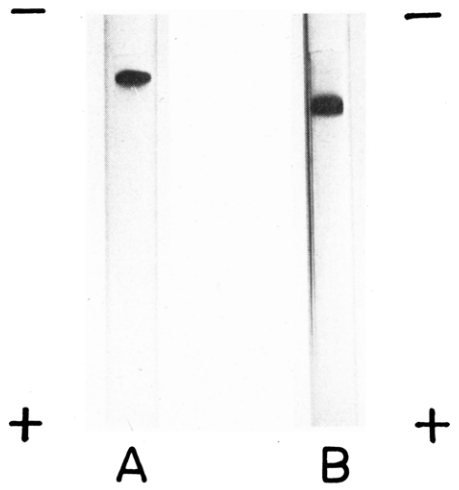

Figure 5. Polyacrylamide gel electrophoresis pattern of (A) gossypin and (B) congossypin in 0.12 M phosphate buffer, pH 8.0.

has been reported for congossypin.

The proteins did not contain any phosphorus and were free from bound and free gossypol impurities. The absence of phosphorus in proteins suggests that they were free from nucleic acid contamination.

Thus, the method described in this investigation could yield homogeneous gossypin and congossypin from cottonseed. It has the following advantages over other methods: (i) The same extract is used for the isolation of gossypin and congossypin. (ii) It avoids subjecting the proteins to extreme changes in environment, including pH.

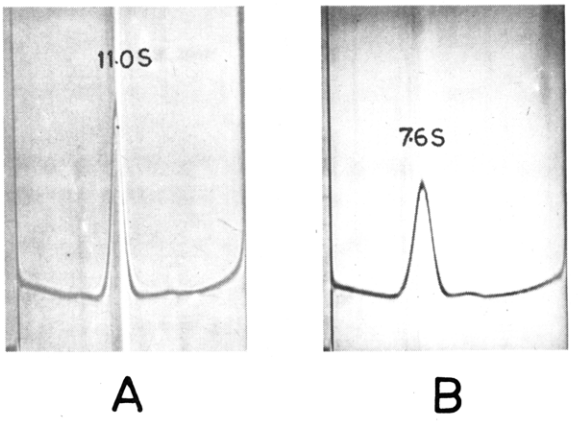

Figure 6. Sedimentation velocity pattern of (A) gossypin and (B) congossypin in 0.05 M Tris-HCl buffer, pH 7.0, containing 1.0 M NaCl. Photographs taken after 36 min of centrifugation for gossypin and 59 min of centrifugation for congossypin at 59780 rpm; bar angle 65°. Sedimentation proceeds from left to right.

(iii) Concentration of the protein is achieved by either cryoprecipitation or ammonium sulfate precipitation. (iv) Large quantities of proteins can be obtained by using preparative columns. (v) The proteins are free from gossypol (free and bound) impurities.

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