Isolation of Proteins from Glanded Cottonseed

A procedure is described whereby intact pigment glands are removed from ground cottonseed kernels by filtration in glycerol prior to protein extraction. Proteins are then separated on a structural basis into storage and nonstorage isolates. Undenatured proteins are recovered with an average yield of 32%, of which 67% are storage proteins. The recovered isolates may be further purified in any desired manner. This procedure should be useful as an initial purification step in laboratory studies of proteins from glanded cottonseeds.

If cottonseed (*Gossypium hirsutum* L.) is to realize its full potential as a source of food, varieties with desirable protein quality characteristics must be developed. Knowledge of the ontogeny, amino acid composition, and heritability of individual cottonseed proteins will be required for development of varieties for specific end uses.

Any technique used to study the proteins of cottonseed must overcome the problem of gossypol, a toxic pigment located within glands distributed throughout the seeds of most cultivated varieties (Bell and Stipanovic, 1977; Vix et al., 1972). Gossypol is water insoluble, but the walls of the pigment glands rupture in most aqueous media, thereby releasing the pigment which then rapidly interacts with proteins. These interactions reduce yields of extractable proteins, modify the pattern of amino acids released by hydrolysis (Damaty and Hudson, 1975a), alter protein solubility characteristics (Damaty and Hudson, 1975b), and inhibit the activity of some enzymes (Myers and Throneberry, 1966; Finlay et al., 1973).

Studies of the proteins and enzyme activities of cottonseeds have been hampered by these detrimental effects of gossypol. The problem can be circumvented by the use of glandless varieties of cottonseed, but this approach precludes evaluation of the more common varieties and the exotic species of *Gossypium*, almost all of which are glanded. Alternatively, the problem of gossypol may be ignored, and glanded seeds extracted with aqueous solutions; there is a possibility that data obtained in this manner will be erroneous. We report here the details of a laboratory procedure by which intact pigment glands are removed from mature ungerminated cottonseeds by filtration in a nonaqueous medium prior to protein extraction. It is possible to separate storage proteins, located in membrane-bound protein bodies (Altshul et al., 1966), and nonstorage proteins with little danger of interference by glandular pigments.

This procedure grew out of a series of modifications of a method (Yatsu and Jacks, 1968) developed for ultrastructural studies of glandless cottonseeds. Using glanded seeds, we compared our procedure with that of Yatsu and Jacks and with an aqueous extraction method (Berardi et al., 1969) suitable for use with glandless and low gossypol cottonseed flours. This aqueous method (Berardi et al., 1969) was designed as the basis for commercial-scale preparation of protein isolates from cottonseed. It is included here only for purposes of comparison, being representative of aqueous methods that have been used to prepare cottonseed proteins for biochemical studies.

MATERIALS AND METHODS

Ground Cottonseed Kernels. Glanded cottonseeds, var. Deltapine 16, were dehulled in a Bauer disc mill. Hulls, fines, and uncracked seeds were removed. The remaining clean kernels were ground in a knife mill (Mitey-Mill, Stur-Dee Health Products, Island Park, N.Y.) for 15–30 s and brushed through a 60 mesh screen. The resulting product was stored at −50 °C over anhydrous $CaSO_4$.

Extraction and Fractionation of Proteins. Each of the three compared procedures separates seed proteins into storage and nonstorage fractions. The procedure of Yatsu and Jacks calls for the homogenization of whole dehulled seeds in glycerol, but by following the published procedure we were unable to achieve any significant breakup of the seeds, much less homogenization. For this reason and for more direct comparisons, we used ground cottonseed kernels with all three procedures.

(*A*) *Aqueous Procedure.* This procedure was carried out as published (Berardi et al., 1969), but at 5 °C instead of room temperature. Ground cottonseed kernels (0.5 g) were stirred for 30 min with 7.5 mL of H_2O. Centrifugation at 17 000*g* for 30 min yielded a supernate containing nonstorage proteins. The residue was extracted for 30 min with 7.5 mL of 0.015 N NaOH and centrifuged at 17 000*g* for 30 min; the resulting extract contained storage proteins.

(*B*) *Yatsu and Jacks Procedure.* Three grams of ground cottonseed kernels were thoroughly mixed with 10 mL of glycerol, and centrifuged at 1100*g* for 5 min. The lipid layer, which also contained most of the pigment glands, was removed with a spatula and the opaque liquid transferred to a clean tube and centrifuged at 41 000*g* for 20 min. The supernate (nonstorage proteins) was decanted and diluted with 10 mL of 1 M NaCl. Pelleted protein bodies were washed with 3 mL of glycerol which was subsequently added to the dilute supernate. The protein bodies were then homogenized with 5 mL of 2 M NaCl, centrifuged at 10 000*g* for 20 min, and the pellet discarded. Because of the high viscosity of cold glycerol, all steps prior to NaCl dilutions were conducted at room temperature; subsequent steps were at 5 °C.

(*C*) *Filtration Procedure.* One gram of ground cottonseed kernels and 3 mL of glycerol were placed on a glass TLC plate resting on a bed of shaved ice. The substances were mixed thoroughly with a spatula and the mixture was transferred to a 15 × 15 cm^2 of Miracloth (cellulosic filtration medium, Calbiochem, San Diego, Calif.). The Miracloth was twice folded in half to form a corner containing the mixture. The mixture was then expressed through the Miracloth with a spatula onto another glass plate. Microscopic examination of the filtrate indicated that all the pigment glands were retained by the Miracloth. The filtrate was transferred into a centrifuge tube. The residue was mixed with 2 mL of glycerol, again expressed through Miracloth, and the filtrate was added to that in the tube. After this procedure was repeated twice, the residue was discarded. The combined filtrates (9 mL) were brought to 25 mL volume with cold (5 °C) 0.25 M sucrose and mixed gently. After centrifugation at 20 000*g* for 10 min at 5 °C, the lipid layer was removed with a spatula; the supernate was filtered through Miracloth to remove residual lipid. The filtrate contained the nonstorage proteins of the cottonseed. The pellet, consisting of intact

Table I. Yields and Distribution of Cottonseed Protein[a]

Procedure	Protein yield,[b] %	Protein distribution,[c] %	
		Non-storage	Storage
Aqueous	79 ± 1	25 ± 1	75 ± 1
Yatsu and Jacks	6 ± 2	37 ± 10	63 ± 10
Filtration	32 ± 1	33 ± 3	67 ± 3

[a] Mean values ± standard deviation are from three preparations by each procedure, except for that of Yatsu and Jacks, nine preparations. [b] Percent of total seed meal protein based on N analysis. [c] Percent of recovered protein based on N analysis.

storage protein bodies (Lui and Altschul, 1967), was suspended in 0.25 M sucrose and centrifuged as before. Storage proteins were solubilized by triturating the pellet in 10 mL of 0.015 N NaOH. The solution was clarified by centrifugation at 10 000*g* for 20 min at 5 °C.

Estimation of Protein Content. Nitrogen content of ground kernels and of all isolates was determined with a Coleman Model 29 nitrogen analyzer. Protein content was calculated using conversion factors of 5.05 for ground kernels, 4.58 for storage isolates, and 5.22 for nonstorage isolates as determined by amino acid analysis (unpublished data, this laboratory).

Determination of pH–Solubility Characteristics. Protein isolates were diluted with water to approximately 1 mg/mL. The pH of 3.0-mL aliquots was adjusted with dilute HCl or NaOH to cover the range 2.0 to 11.0 in increments of 1.0 pH except in the areas of minimum solubility, where the increments were 0.3 pH. Each aliquot was then diluted with water to 4.0 mL and centrifuged at 3000*g* for 20 min at room temperature. Soluble protein was estimated by the Lowry procedure modified for use in the presence of interfering material such as glycerol (Bensadoun and Weinstein, 1976). Solubility profiles (Figures 3 and 4) were obtained by plotting protein solubility vs. pH; plotted values are means from four replications of the isolation procedures.

RESULTS AND DISCUSSION

Protein Yields and Distribution. Protein yields and distribution for isolates from the three methods appear in Table I. The low yield of the Yatsu and Jacks procedure was probably due to an aggregation of protein bodies with spherosomes (lipid droplets) and pigment glands in the lipid layer formed during the initial centrifugation. Presence of protein bodies in this layer was confirmed by both light microscopy and N analysis. We attempted to increase dispersal of protein bodies by allowing original mixture to stand from 30 to 60 min with occasional stirring, and by mixing the meal and glycerol vigorously in a laboratory homogenizer. Neither modification improved the yield. Because of the low yield and the variable distribution encountered with the Yatsu and Jacks procedure, it was omitted from further comparison. Yield from the nonaqueous filtration procedure was less than that of the aqueous method because of the amount of N-rich residue following the gland-filtration step. We think some sacrifice of yield is worthwhile to minimize time spent in filtration and to avoid possible mechanical rupture of glands while expressing gland-rich material through Miracloth.

Distributional differences between the filtration and aqueous procedures may be attributed to gossypol-induced precipitation of nonstorage proteins during the first extraction of the aqueous method. This precipitation thereby enriched the "storage" protein fraction. Because a non-

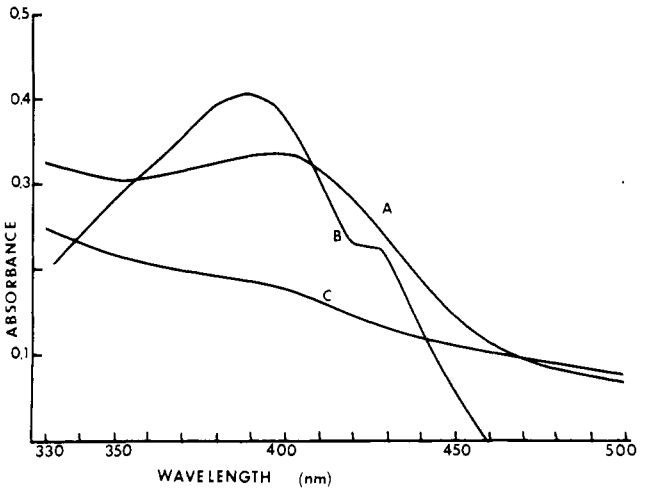

Figure 1. Absorbance spectra of nonstorage protein isolates and protein–gossypol complex: (A) from aqueous procedure, diluted to 0.3 mg/mL with 0.015 N NaOH; (B) bovine serum albumin–gossypol complex, 10 mg/mL in phosphate buffer (after Lyman et al., 1959); (C) from filtration procedure, diluted to 0.3 mg/mL with 0.015 N NaOH.

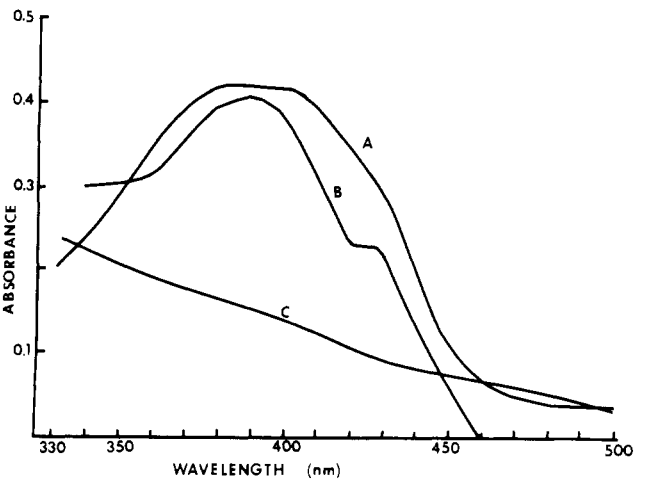

Figure 2. Absorbance spectra of storage protein isolates and protein–gossypol complex: (A) from aqueous procedure, diluted to 0.26 mg/mL with 0.015 N NaOH; (B) bovine serum albumin–gossypol complex, 10 mg/mL in phosphate buffer (after Lyman et al., 1959); (C) from filtration procedure, diluted to 0.37 mg/mL with 0.015 N NaOH.

aqueous medium was used in the filtration procedure, protein separation was on a structural or physical basis, and protein bodies were separated from the nondiscrete nonstorage species.

Gossypol Content of Fractions. Proteins extracted by the aqueous procedure were gray-brown and were difficult to dissolve. Such characteristics represent a serious drawback to the use of aqueous procedures for the isolation of proteins from glanded seed. Lyman et al. (1959) demonstrated that interaction of bovine serum albumin and gossypol produced a characteristic ultraviolet-visible absorption spectrum with a broad peak centered at 390 nm (Figures 1 and 2, curve B). Similar spectra confirmed the presence of gossypol in protein isolates prepared by the aqueous procedure (Figures 1 and 2, curve A). In contrast, proteins from the nonaqueous filtration procedure were light colored, dissolved easily, and exhibited essentially no spectrophotometric evidence of gossypol contamination (Figures 1 and 2, curve C).

pH–Solubility Characteristics. For isolates prepared by the aqueous procedure, the pH of minimum solubility

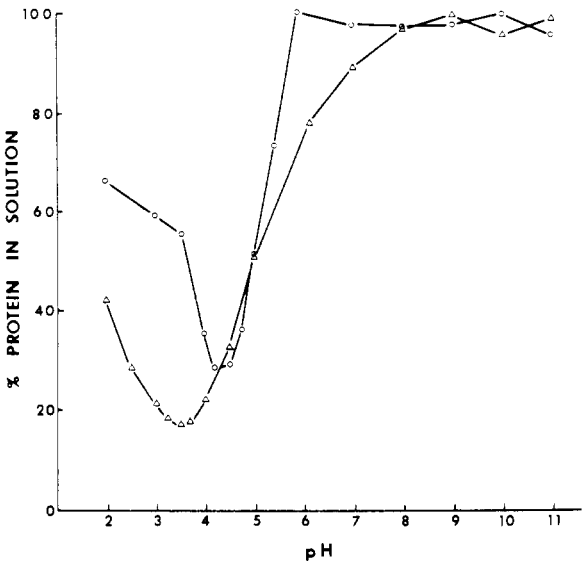

Figure 3. pH–solubility profile of nonstorage protein isolates at 0.75 mg/mL: (△) isolate prepared by aqueous procedure; (○) isolate prepared by filtration procedure.

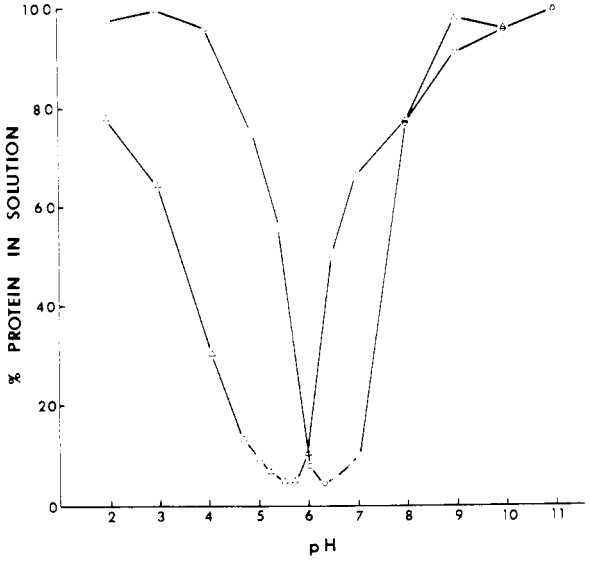

Figure 4. pH–solubility profile of storage protein isolates at 0.75 mg/mL: (△) isolate prepared by aqueous procedure; (○) isolate prepared by filtration procedure.

was about one unit lower than that for isolates prepared by the nonaqueous filtration procedure (Figures 3 and 4). These results are comparable with those of Damaty and Hudson (1975b), who described similar acid shifts associated with increased levels of gossypol in seed of *G. barbadense*. Gossypol forms Schiff bases with accessible free amino groups of a protein, thereby decreasing the basic nature of the molecule (Conkerton and Frampton, 1959). Baliga (cited by Lyman et al., 1959) found that free ε-amino groups of lysine comprise the major portion of available sites on cottonseed protein; the free amino groups of arginine and N-terminal amino acids may also be somewhat vulnerable.

SUMMARY

We have developed a laboratory-scale procedure whereby native proteins that are low in pigment contamination and retain their original properties can easily be isolated from glanded cottonseeds. The basis of the procedure is the removal of intact pigment glands before proteins are extracted and separated, on a structural basis, into storage and nonstorage isolates. These isolates are then amenable to further purification and analysis by any desired technique. The filtration procedure, which constitutes a logical first step in the isolation of unmodified protein species from cottonseed, should be of value in laboratory studies of cottonseed proteins and their biochemistry, and in the development and screening of varieties with improved nutritive properties.

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LITERATURE CITED

Altschul, A. M., Yatsu, L. Y., Ory, R. L., Engleman, E. M., *Ann. Rev. Plant Physiol.* **17**, 113 (1966).
Bell, A. A., Stipanovic, R. D., *Proc. Beltwide Cotton Prod. Res. Conf.*, in press (1977).
Bensadoun, A., Weinstein, D., *Anal. Biochem.* **70**, 241 (1976).
Berardi, L. C., Martinez, W. H., Fernandez, C. J., *Food Technol.* **23**, 1305 (1969).
Conkerton, E. J., Frampton, V. L., *Arch. Biochem. Biophys.* **81**, 130 (1959).
Damaty, S., Hudson, B. J. F., *Proc. Nutr. Soc.* **34**, 48A (1975a).
Damaty, S., Hudson, B. J. F., *J. Sci. Food Agric.* **26**, 1667 (1975b).
Finlay, T. H., Dharmgrongartama, E. D., Perlmann, G. E., *J. Biol. Chem.* **248**, 4827 (1973).
Lui, N. S. T., Altschul, A. M., *Arch. Biochem. Biophys.* **121**, 678 (1967).
Lyman, C. M., Baliga, B. P., Slay, M. W., *Arch. Biochem. Biophys.* **84**, 486 (1959).
Myers, B. D., Throneberry, G. O., *Plant Physiol.* **41**, (1966).
Vix, H. L. E., Gardner, H. K., Lambou, M. G., Rollins, M. L., in "Symposium: Seed Proteins", Avi Publishing Co., Westport, Conn., 1972, p 212.
Yatsu, L. Y., Jacks, T. J., *Arch. Biochem. Biophys.* **124**, 466 (1968).

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