Albumin Storage Protein and Allergens in Cottonseeds

Richard J. Youle[1] and Anthony H. C. Huang*

Cottonseed contains three major types of proteins having sedimentation values of 2S, 5S, and 9S and existing in equal amounts. Protein bodies isolated from the cottonseed also contain these three major proteins in similar proportions. The 5S and 9S proteins are typical globulin storage proteins and have similar amino acid compositions. The 2S proteins are albumins and are also storage protein as judged by their amino acid composition, developmental properties, and high amount in the seed. The 2S proteins are distinct from the 5S and 9S proteins in solubility, amino acid composition, sedimentation values, and sodium dodecyl sulfate gel electrophoretic patterns. The 2S storage albumin proteins are shown by $NaDodSO_4$ gel electrophoresis, immuno-cross-reactivity, and amino acid composition to be identical with the cottonseed allergens.

The utilization of plant proteins to supplement diet has been receiving increased attention partly due to the expanding demand on dietary proteins around the world. An important source of these plant proteins is the protein byproduct of oilseeds after the oil has been extracted for industrial utilization. Cottonseed is an important oilseed to provide the protein byproduct because of the enormous amount of cottonseeds required for fiber production (see review by Cater et al., 1977). In the study of the composition of cottonseed protein, the protein was extracted by industrial processing and categorized arbitrarily into storage protein and nonstorage protein (Berardi et al., 1969; Cater et al., 1977; King and Lamkin, 1977). However, the chemical characteristics of these protein components of the cottonseed in relation to their in vivo properties and functions are unknown.

In seeds other than those of Gramineae, the classical storage protein is globulins of high molecular weight (Osborne, 1924; Danielsson, 1949). Albumin storage protein of low molecular weight occurring in abundance has recently been described in castor bean (Youle and Huang, 1976, 1978a). The storage protein is localized in single-membrane organelles called protein bodies. In seeds of certain species like cotton, the protein body contains an amorphous protein matrix (Yatsu and Jacks, 1968). In seeds of other species such as castor bean, the protein body contains a large protein crystalloid embedded in the amorphous protein matrix (Ory et al., 1968; Tully and Beevers, 1976; Youle and Huang, 1976). In castor bean, whereas the globulin storage protein is localized in the protein crystalloid of the protein body, the albumin storage protein is restricted to the amorphous protein matrix (Youle and Huang, 1976, 1978a) of the organelle.

Many seeds, especially oilseeds, contain potent allergens of protein nature (see review by Berrens, 1971, and Spies, 1974; Daussant et al., 1976). Although the chemical properties of the oilseed allergens have been extensively studied, the cellular aspects of these proteins such as their in vivo function, subcellular localization, and percent of the total seed protein have not been investigated until very recently. The castor bean allergen is identified as the low-molecular-weight albumin storage protein in the amorphous matrix of the protein bodies (Youle and Huang, 1978b). The cottonseed contains the best-studied oilseed allergens (Spies, 1941; Spies et al., 1951, 1960), but the in vivo features of the cottonseed allergens have not been elucidated. Whether or not the cottonseed allergens serve the same in vivo function as the castor bean allergen is unknown.

To further the knowledge on seed storage proteins and seed allergens, we have chosen to study the cottonseed for its increasing importance in human consumption and for its representation of those seeds whose protein bodies contain only amorphous protein matrix without protein crystalloids. In this paper, we present a characterization of the major proteins in cottonseed. The in vivo roles of the various proteins are identified and the cottonseed allergens are shown to be a major albumin storage protein.

MATERIALS AND METHODS

Preparation of Protein Fractions. Three grams of dry dehulled cottonseed (*Gossypium hirsutum* L. cv. SJ-2) were ground in 100 mL of 1 M NaCl, 0.035 M sodium phosphate buffer, pH 7.5 for 20 min in a VirTis 45 homogenizer. The crude extract was centrifuged at 10000*g* for 30 min and the supernatant fraction beneath the fat layer was used as the total protein extract.

Protein bodies from dry cottonseed were isolated by differential centrifugation in glycerol according to the method of Yatsu and Jacks (1968). Five grams of dehulled cottonseed were ground in 15 mL of glycerol at low speed in a VirTis homogenizer for 5 min. The homogenate was filtered through cheesecloth and centrifuged at 41000*g* for 20 min. The resulting pellet was resuspended in 5 mL of glycerol and recentrifuged as before. The pellet contained the protein bodies and was resuspended in glycerol.

Cottonseed allergens (CS-1A) (Spies et al., 1951) were generously supplied by Dr. J. R. Spies.

Determination of Sedimentation Values by Ultracentrifugation. Protein fractions were prepared for ultracentrifugation by dialyzing against 0.035 M sodium phosphate buffer, pH 7.5, and 1 M NaCl. Purified protein bodies in glycerol were diluted in this buffer; the dilution lysed the organelles and solubilized the protein. After lysis of the organelles, the globoids and membranes were removed by centrifugation at 10000*g* for 30 min. Since catalase dissociated into its subunits in 1 M NaCl, the enzyme was dissolved in the buffer without NaCl.

Sedimentation analyses were performed by sucrose gradient centrifugation according to Martin and Ames (1961) with the modification of Hill and Breidenbach (1974). Each 1-mL protein sample was applied on top of a linear density gradient composed of 34 mL of 5–30% (w/w) sucrose. The gradient solution also contained 0.035 M sodium phosphate buffer, pH 7.5, and 1 M NaCl. In the centrifugation of catalase, NaCl was omitted in the gradient solution. The gradient was centrifuged at 25000 rpm for 24 h in a Beckman L2-65B ultracentrifuge using

Biology Department, University of South Carolina, Columbia, South Carolina 29208.

[1]Present address: Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, MD 20014.

0021-8561/79/1427-0500$01.00/0

a SW-27 rotor. The gradient was fractionated into 1-mL fractions and the protein was assayed according to Lowry et al. (1951) with BSA as the standard. Myoglobin (type II, Sigma) and bovine liver catalase (type C-10, Sigma) were used as markers for sedimentation values. Catalase was assayed as described (Lück, 1965) and myoglobin was assayed by the Lowry method.

$NaDodSO_4$ Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was performed in 15% polyacrylamide gels with $NaDodSO_4$ and a discontinuous buffer system (Weber and Osborne, 1975). Protein samples were incubated at room temperature for 30 min, or at 100 °C for 2 min in 0.01 M Tris-HCl buffer, pH 6.8, and 1% $NaDodSO_4$. Molecular weight standards were those described earlier (Youle and Huang, 1976).

Immunological Techniques. Each of two 5-lb New Zealand white rabbits was injected in the foot pads and the back subcutaneously with 2 mg of cottonseed allergens mixed in Freund's complete Adjuvant. After 1 week, it was injected in the back subcutaneously with 2 mg of cottonseed allergens mixed with Freund's incomplete Adjuvant. The rabbits were bled 10 days after the second injection and the γ-globulins were purified by the procedure of Kendall (1938). The γ-globulins were dialyzed against 0.01 M sodium phosphate buffer, pH 7.5, 0.05 M NaCl, and after centrifugation at 10000g for 30 min, the supernatant fraction was retained. γ-Globulins were also prepared by an identical procedure from untreated rabbits as controls.

Double diffusion tests were performed according to Ouchterlony (1968). Equal amounts of protein samples to be tested were applied to each well except that twice the amount of the total cottonseed proteins were used.

Protein Hydrolysis and Amino Acid Analysis. After dialysis against water and lyophilization, the protein samples were hydrolyzed to amino acids with 4 N methanesulfonic acid in vacuo. The procedure that preserved tryptophan was followed (Simpson et al., 1976). The protein samples were reduced with dithiothreitol for half-cysteine analysis. Amino acid analyses were performed on a Beckman 120-C instrument.

Germination. Cottonseeds were soaked overnight in running tap water and germinated in moist vermiculite at 30 °C in darkness. The cotyledons at various stages were ground with a mortar and pestle in $NaDodSO_4$ gel electrophoresis buffer (Youle and Huang, 1976). All the extracts were made to the same volume with buffer. The extracts were centrifuged at 5000g for 10 min and the supernatant fractions were used for gel electrophoresis.

RESULTS

Sedimentation Profile. The total cottonseed proteins extracted in 1 M NaCl were analyzed by sucrose gradient centrifugation to determine the relative amounts and the sedimentation values of the major proteins. The sedimentation centrifugation revealed three major peaks of protein with sedimentation values of 2S, 5S, and 9S (Figure 1a). Each of the three protein peaks represented approximately one-third of the total protein.

The proteins in isolated cottonseed protein bodies were also extracted with 1 M NaCl and analyzed by sucrose gradient centrifugation. A protein profile very similar to that of the total cottonseed protein was found (Figure 1b). We suggest that the three major cottonseed proteins are localized in the protein bodies because (1) the protein profiles of the total cottonseed protein and of the isolated protein bodies in the sucrose gradients are very similar, (2) the $NaDodSO_4$ gel protein profile of the total cottonseed proteins and that of the isolated protein bodies

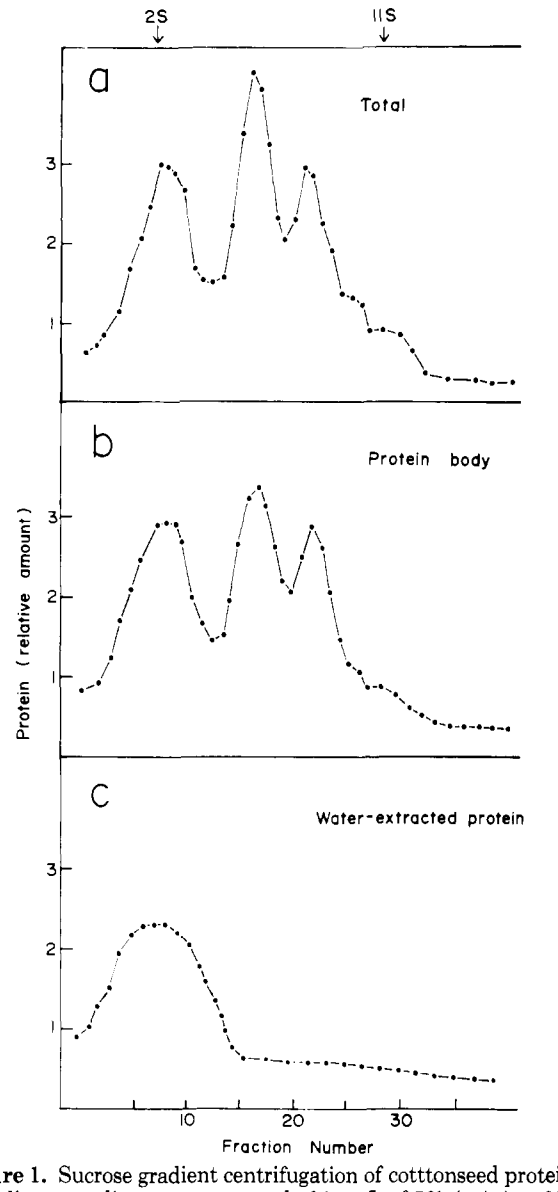

Figure 1. Sucrose gradient centrifugation of cotttonseed proteins. Each linear gradient was composed of 34 mL of 5% (w/w) to 30% sucrose in 1 M NaCl. Centrifugation was carried out in a Beckman SW27 rotor for 24 h at 25000 rpm. Myoglobin (2.04S) and catalase (11.3S) were used as markers of sedimentation values.

are identical (see latter sections describing Figure 2A and Figure 3), and (3) a similar conclusion supported with more detailed data has been reported in castor bean (Youle and Huang, 1976, 1978a,b). However, definite evidence is required to show that our suggestion is completely valid. In our subsequent study on the properties of the three major proteins, especially the 2S proteins, which are the focal points of this paper, we obtained the proteins directly from the cottonseeds instead of from the isolated protein bodies.

The various isolated protein fractions were free from bound gossypol since none of them exhibited spectrophotometric evidence of gossypol contamination (King and Lamkin, 1977).

Solubilities. The solubility of the three major protein fractions was investigated. Cottonseed protein was extracted in dilute buffer as described before except that NaCl was omitted. The proteins extracted were thus soluble in low ionic strength media and were analyzed by sucrose gradient centrifugation (Figure 1c). Only one protein peak was found which was at the same position as that of the 2S protein peak of the total seed protein

Table I. Percentage Composition of Amino Acids of Cottonseed Proteins

	protein fractions		
	2S	5S	9S
Trp	1.68	Tr	0.6
Lys	7.46	4.03	2.8
His	2.26	2.92	2.1
Arg	10.32	7.37	9.1
Cys	7.74	1.81	3.2
Asp	8.37	9.55	10.6
Thr	2.86	4.54	3.4
Ser	4.05	7.25	5.3
Glx[a]	27.23	16.74	18.5
Pro	2.96	3.77	3.5
Gly	8.69	8.38	9.0
Ala	4.39	5.64	6.7
Val	1.68	6.62	5.7
Met	1.80	1.82	2.0
Ile	1.53	3.78	3.5
Leu	2.55	6.78	6.6
Tyr	2.86	3.29	1.8
Phe	1.55	1.80	5.4

[a] Glx, glutamate and glutamine.

(Figure 1a,b). The 2S proteins thus appear to be albumins in solubility whereas the 5S and 9S proteins are globulins.

The solubility of the isolated 2S and 9S proteins was also compared. The two protein fractions obtained from sucrose gradients in 1 M NaCl were dialyzed against water for 24 h. The protein fractions were then centrifuged at 10000*g* for 30 min, and the amount of soluble protein in the supernatant and insoluble protein in the pellet were determined. Of the 2S proteins, 69% remained soluble in water, whereas only 8% of the 9S proteins remained soluble. This finding again suggests that the 2S proteins are albumins whereas the 9S proteins are globulins.

Amino Acid Composition. The amino acid compositions of the 2S, 5S, and 9S protein fractions were determined. The amino acid compositions of all three protein fractions exhibit characteristics of storage proteins with high glutamate/glutamine and arginine contents (Table I). The 5S and 9S proteins, both being globulins, have very similar amino acid compositions. The 2S proteins, being albumins, have a different amino acid content. They have higher glutamate/glutamine, arginine, and cysteine contents than the 5S and 9S proteins.

2S Proteins as Storage Proteins. The 5S and 9S proteins appear to be typical seed storage proteins because of their abundance in cottonseed, high amide amino acid content, insolubility in water, high molecular weight, and presence in protein bodies. The 2S proteins are also abundant in the cottonseed, present in protein bodies, and enriched in amide amino acids. However, they are not typical seed storage proteins since they are water soluble and have low molecular weight. A similar unusual type of albumin storage protein has recently been reported in castor bean (Youle and Huang, 1978a). Whether or not the cottonseed 2S proteins are storage proteins and how they might be related to the castor bean albumin storage protein were investigated.

The protein body proteins and the 2S proteins isolated from the total cottonseed by sucrose gradient centrifugation were compared by $NaDodSO_4$ polyacrylamide gel electrophoresis (Figure 2A,B). In 15% acrylamide gels, the protein body proteins were resolved into several protein bands of high molecular weight and three protein bands of low molecular weight around 15000. The isolated 2S proteins consisted of three protein bands of low molecular weight as those of the total protein body proteins. Striking similarity in the $NaDodSO_4$ gel protein profile was observed between the isolated protein body proteins (Figure 2A) and the total cottonseed proteins (first column, Figure 3).

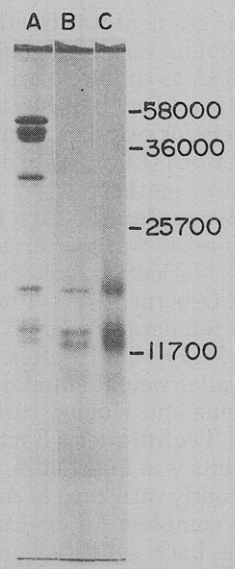

Figure 2. Photograph of 15% $NaDodSO_4$ polyacrylamide gels after electrophoresis of the cottonseed protein body proteins (A), the purified cotton seed 2S proteins (B), and the cottonseed allergens (C). Numbers on the right are estimated molecular weight.

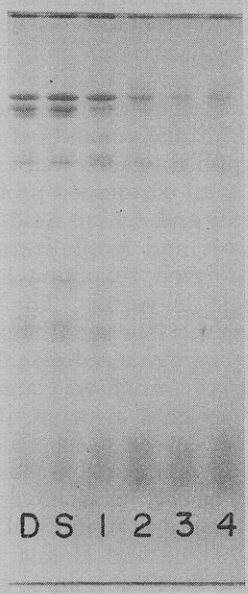

Figure 3. Photograph of a 15% $NaDodSO_4$ polyacrylamide slab gel electrophoresis of the total extracts of cottonseed cotyledons after various days of germination. D and S denote dry and soaked seeds, respectively.

Should the 2S proteins be storage proteins, they would be mobilized and decomposed rapidly during germination. To investigate this possibility, the total cottonseed proteins in the cotyledons were extracted at different stages of germination and analyzed by $NaDodSO_4$ acrylamide gel electrophoresis (Figure 3). The three protein bands of low molecular weight comprising the 2S proteins were degraded very rapidly during germination. They disappeared completely after 2 days of germination. The finding further supports the premise that the 2S proteins are storage proteins.

Cottonseed Allergen Proteins. Cottonseeds contain a low molecular weight, water-soluble allergen which has been extensively studied (Spies, 1941; Spies et al., 1951,

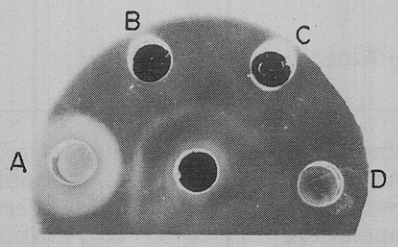

Figure 4. Photograph of Ouchterlony double diffusion tests of rabbit γ-globulins prepared against cottonseed allergens. Central well, rabbit γ-globulins; well A, total proteins of cottonseed; well B, cottonseed 2S proteins; well C, cottonseed allergens; well D, cottonseed 9S proteins. Wells B and C contained the same amount of proteins and wells A and D contained twice the amount. Well A had a ring of unknown precipitation surrounding it. A minor second precipitin line, indicating antigenetically cross-reactive between the samples in wells A, B, and C, appeared very close to the central well.

1960). The relationship between the allergen and the 2S storage proteins was investigated.

The protein pattern of the 2S proteins in $NaDodSO_4$ polyacrylamide gels was compared with that of the cottonseed allergen. Figure 2 shows that the patterns of protein bands of the two protein preparations appear to be identical.

Rabbit γ-globulins raised against the cottonseed allergen were used to test the antigenic relationship between the allergen and the 2S proteins. In the Ouchterlony double diffusion test (Figure 4), the γ-globulins reacted with the total cottonseed protein, the 2S protein, and the allergen. The precipitin line between each of the above protein preparations and the γ-globulins was continuous. No reaction was observed with the 9S globulins. The allergens and the 2S storage proteins are thus antigenically indistinguishable in the present test. Besides the immunological test and the gel pattern, we also found that the amino acid composition of the 2S proteins (Table I) is very similar to that of the allergen (Spies et al., 1951).

DISCUSSION

The cottonseed 2S albumins are shown to be present in the protein bodies, to constitute one-third of the total seed protein, to degrade rapidly during germination, and to have an amino acid composition characteristics of storage proteins. Clearly the cottonseed 2S albumins are storage proteins, supplying amino acids to the growing embryonic axis during germination. The properties of the 2S albumin storage proteins are identical with those of the cottonseed "nonstorage protein" categorized arbitrarily based on molecular weight and solubility (reviewed by Cater et al., 1977). Our 5S and 9S globulins are equivalent to the "storage protein" of cottonseed (reviewed by Cater et al., 1977). The "nonstorage protein" and "storage protein" of cottonseed should now be recategorized since they are actually both storage proteins.

The cottonseed 2S proteins appear to be very similar to the albumin storage proteins recently reported in castor bean (Youle and Huang, 1978a) as far as the molecular weight, solubility, and amino acid composition are concerned. Furthermore, they both possess immunological identities and chemical properties similar to those of the closely related cottonseed and castor bean allergens, respectively. In fact, a comparison of the amino acid composition of the major storage proteins in cottonseed and castor bean (Youle and Huang, 1978a) shows a greater similarity between the 2S storage proteins of these two taxonomically unrelated plant species than between the 2S proteins and the globulin storage proteins of each plant species. The present findings extend our previous work on castor bean and indicate that abundant seed 2S albumin storage proteins of characteristic properties are not unique to only one plant species or those species having protein crystalloids in the protein bodies.

ACKNOWLEDGMENT

We thank J. R. Spies for several inspiring discussions and a generous supply of the invaluable CS-1A protein fraction, and R. H. Hardwick of Producer Cotton Oil Co. for a generous supply of cottonseed.

LITERATURE CITED

Berardi, L. C., Martinez, W. H., Fernandez, C., *J. Food Technol.* **23**, 75 (1969).
Berrens, L., *Monogr. Allergy* 7, 74 (1971).
Cater, C. M., Mattil, K. F., Meinke, W. W., Taranto, M. V., Lawhon, J. T., *J. Am. Oil Chem. Soc.* **54**, 90A (1977).
Danielsson, C. E., *Biochem. J.* **44**, 387 (1949).
Daussant, J., Ory, R. L., Layton, L. L., *J. Agric. Food Chem.* **24**, 103 (1976).
Hill, J. E., Breidenbach, R. W., *Plant Physiol.* **53**, 742 (1974).
Kendall, F. E., *Cold Spring Harbor Symp. Quant. Biol.* **6**, 376 (1938).
King, E. E., Lamkin, G. E., *J. Agric. Food Chem.* **25**, 1211 (1977).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).
Lück, H., "Methods of Enzymatic Analysis", Bergmeyer, H. U., Ed., Academic Press, New York, 1965, p 885.
Martin, R. G., Ames, B. N., *J. Biol. Chem.* **236**, 1372 (1961).
Ory, R. L., Yatsu, L. Y., Kircher, H. W., *Arch. Biochem. Biophys.* **264**, 255 (1968).
Osborne, T. B., "The Vegetable Proteins", 2nd ed, Longman's, Green, London, 1924, Chapter 4.
Ouchterlony, O., "Handbook of Immunodiffusion and Immunoelectrophoretic Techniques", Ann Arbor Humphrey Science Publishers, Ann Arbor, MI, 1968 p 21.
Simpson, R. F., Neuberger, M. R., Liu, T. Y., *J. Biol. Chem.* **251**, 1936 (1976).
Spies, J. R., *J. Am. Chem. Soc.* **63**, 1166 (1941).
Spies, J. R., *J. Agric. Food Chem.* **22**, 30 (1974).
Spies, J. R., Coulson, E. J., Chambers, D. C., Berton, H. S., Stevens, H., Shimp, J. H., *J. Am. Chem. Soc.* **73**, 3995 (1951).
Spies, J. R., Bernton, H. S., Chamber, D. C., *J. Allergy* **31**, 162 (1960).
Tully, R. E., Beevers, H., *Plant Physiol.* **58**, 710 (1976).
Weber, K., Osborne, M., in "The Proteins", Neurath, H., Hill, R. L., Ed., Academic Press, New York, 1975, p 180.
Yatsu, L. Y., Jacks, T. Y., *Arch. Biochem. Biophys.* **124**, 466 (1968).
Youle, R. J., Huang, A. H. C., *Plant Physiol.* **58**, 703 (1976).
Youle, R. J., Huang, A. H. C., *Plant Physiol.* **61**, 13 (1978a).
Youle, R. J., Huang, A. H. C., *Plant Physiol.* **61**, 1040 1978b).

Received for review September 11, 1978. Accepted December 14, 1978. This research was supported by National Science Foundation Grant PCM 77-17679.