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Nature and Patterns of Proteins during Cotton Seed Development

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ABSTRACT

Patterns of accumulation and ontogenetic relationships among proteins of cotton (*Gossypium hirsutum* L.) seeds were examined between 10 days postanthesis and maturity (56 days). Total and extractable nitrogen contents were determined; alkali- and water-soluble proteins were assayed quantitatively and electrophoretically. Two alkali-soluble proteins present in the electrophoretogram of mature embryos first appeared at 21 days postanthesis; most of the final profile was established by 28 days. Except for minor changes centering around the 7th week of development, the pattern from 28 days to maturity was marked by intensification of bands. The quantity of water-soluble nitrogen increased through the first 21 days of development, then declined until 42 days, when it again began to increase; it reached its highest level at maturity. There was evidence of a high peptide content 7 weeks postanthesis.

Although seed development in cotton (*Gossypium hirsutum* L.) has been described in several reports (5, 6, 16, 18), studies of the pattern of protein accumulation during cotton seed development are lacking. Analyses of dry matter and mineral accumulation and distribution within the developing cotton fruit indicated that the carpel walls contribute a significant proportion of their nitrogen to developing seed (16, 18). Significant incorporation of photosynthetically assimilated ^{14}C has been measured throughout seed development (2).

During the first few weeks of cotton seed development, nearly half of the seed nitrogen is found in the nonprotein pool (6). Although the amino acids in this nonprotein nitrogen pool show little similarity to the amino acid profile of mature cotton seeds, they probably reflect the composition of the amino acids supplied to the developing seeds. Not until midway in seed development does the amino acid composition of the cotton seed begin to resemble the mature seed profile (6).

Studies of seed protein development have commonly measured crude protein accumulation and/or dynamic aspects of amino acid composition (6, 14, 20) while others have investigated structural aspects of seed protein development (4, 7) or the nuclear regulation of protein synthesis (5). Several investigators, however, have examined the development of protein fractions through the developmental changes revealed by electrophoresis or gradient centrifugation of seed proteins. Using electrophoretic separations, Hall *et al.* (10) found that the protein complement of beans changed markedly during development and that storage protein synthesis was underway by the time the seeds were about two-thirds of their mature length. Seed proteins of soybeans can be separated into classes according to sedimentation by density gradient centrifugation (12). These protein classes predominate at different stages of development and can be further subdivided by electrophoresis under appropriate conditions (11, 12). Differential solubility of proteins can also be employed to separate seed proteins into classes, the development of which can be further investigated with other techniques (3). Temporal differences in accumulation rates and quantities of various protein fractions appear to be common, as do generic differences for seed proteins.

We have applied these procedures to an investigation of seed protein accumulation in cotton, a species that has potential as a source of food protein and has previously been used in studies of developmental biochemistry (13). We report here the accumulation of and developmental changes in alkali- and water-soluble proteins as revealed by changes in electrophoretic patterns during the period of seed formation.

MATERIALS AND METHODS

Flowers of cotton (*G. hirsutum* L. cv. Stoneville 7A glandless) were tagged at anthesis in field plots at Stoneville on July 27, 1977. Tagged bolls (fruit) were harvested first at 10 days postanthesis, and then weekly between 14 and 56 days postanthesis. Each harvest consisted of from 3 to 10 bolls from each of four replications.

Harvested bolls were kept on shaved ice until they were dissected to obtain the developing seeds. The seeds were weighed and extracted on the day of harvest. Ten- and 14-day seeds were not decorticated before extraction since, because of their small size and the tight bond between the integuments, it was practically impossible to do so; seeds from subsequent harvests were decorticated. The experimental materials from the 21-day postanthesis harvest and thereafter represented enbryonic tissue only, while those from the 10- and 14-day harvests were complete seeds.

Nitrogen contents were determined before protein extraction by triplicate analyses on a Coleman nitrogen analyzer.[2] Seeds and embryos for protein extraction were frozen at −70 C and ground in a chilled mortar. Extractions and centrifugations were conducted at 4 C. The ground material was extracted with 4 volumes (v/w) of water for 15 min and was centrifuged at 29,000*g* for 20 min. The aqueous extract was decanted and the pellet was again extracted with water and centrifuged as before. Aqueous extracts were pooled. The pellet from the second aqueous extraction was then suspended in 4 volumes of 0.015 N NaOH for 15 min, centrifuged, decanted, and reextracted as before; the NaOH extracts were combined. Nitrogen contents of the aqueous and alkali extracts were determined as above. Residues following the NaOH extractions were discarded.

Aliquots of the aqueous extracts of the 14- and 49-day samples were adjusted to a final concentration of 10% (w/v) trichloroacetic acid by addition of a 50% solution of trichloroacetic acid. The preparations were kept overnight at 4 C and centrifuged at 29,000*g*

[1] Mississippi Agriculture and Forestry Experiment Station cooperating.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

for 20 min. Nitrogen contents of the supernatant and pellet were determined as above.

Samples (80 μg protein) of the aqueous extracts were electrophoresed in 25-mm-thick 2.5 to 27% linear gradient polyacrylamide gel slabs in a pH 9 discontinuous Tris-sulfate-borate system at 5 C (1). Samples (20 μg protein) of the NaOH extracts were electrophoresed in 2.5-mm-thick 7.5% polyacrylamide gel slabs with 0.1% SDS in pH 7.2 phosphate buffer at 25 C using the method of Weber and Osborn (21). Protein bands were stained with Coomassie blue G-250 (8). Molecular weights of alkali-soluble polypeptides were estimated from the SDS electrophoresis data using plots of log mol wt versus mobility (21). Migration distances were measured by scanning slices of stained SDS gel slabs at 570 nm in a gel-scanning attachment fitted to a Pye Unicam SP1700 spectrophotometer. Standard marker proteins used were BSA, phosphorylase *a* (muscle), pyruvate kinase (muscle), aldolase (muscle), carbonic anhydrase, and myoglobin. Electrophoretic data are presented diagrammatically using mean values of relative mobility from four or more replicate separations at each date. This format permits clear visualization of temporal changes in protein patterns which might otherwise be obscured by variations in length of dye front migration between gels.

RESULTS AND DISCUSSION

Seed Nitrogen. Data for fresh weight and nitrogen controls of developing cotton embryos are summarized in Table I. The nitrogen contents reflect a pattern of accumulation that corresponds closely with similar data from *G. hirsutum* cultivars in different years at this location (18; unpublished results). The continual accumulation of nitrogen throughout the period of boll and seed development and the accelerated uptake between 3 and 6 weeks postanthesis appear to be characteristic of the species. Although environmental conditions may temporarily perturb the pattern, resulting in lowered nitrogen content of the mature seed (17, 18), the basic pattern of accumulation remains fairly stable.

Water-soluble Nitrogen. The initially small amount of nitrogen in the young developing seeds and embryos was predominantly water-soluble (Fig. 1). Previous work (6) indicated a pattern of nonprotein nitrogen accumulation and depletion similar to our curve for water-soluble nitrogen from 10 to 35 days postanthesis. We found that only 30% of the nitrogen in the aqueous extract was precipitated by trichloroacetic acid at 14 days. The 200 μg nitrogen/seed remaining in trichloroacetic acid solution was close to the amount of non-protein nitrogen (215 μg) per 14-day seed extracted with acid-alcohol (6). Although treatment with trichloroacetic acid does not distinguish peptides from either proteins or free amino acids, it does demonstrate that most of the water-soluble nitrogen at 14 days postanthesis was not protein. Electrophoretic analyses strongly support the interpretation that most of the nitrogen in the aqueous extract was nonprotein during the initial 3 weeks of seed development. Not until 21 days postanthesis were we able to detect any protein bands in the aqueous extracts, even after extensive sample concentration and inordinately large sample loads (Fig. 2).

Numerous protein bands were discernible at 21 days (Fig. 2); the pattern of their distribution in the gels was fairly stable throughout the next 2 weeks. There were few changes in the number and relative mobilities of the bands, although several new bands had appeared and the intensity of others had diminished by 35 days. The complexity of the profile had increased markedly by 42 days, when it contained more bands than at any other time during development. The profile was different at each sampling

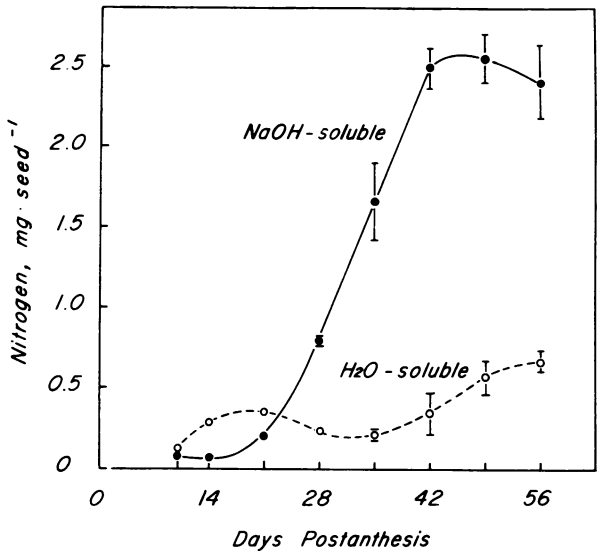

FIG. 1. Amount of nitrogen extracted from cotton embryos by water (○) and by 0.015 N NaOH (●) from 10 to 56 days postanthesis. Vertical bars denote SE.

Table I. Weight, nitrogen content and distribution of extractable nitrogen of developing cotton embryos.

Age After Anthesis	Fresh Weight[a]		Nitrogen	Extractable Nitrogen	
	Seed	Embryo	per Embryo[a]	NaOH-Soluble	H_2O-Soluble
Days	mg		μg	% of extracted N	
10	42.3 ± 2.1	n.d[b]	199 ± 25[c]	36.7	63.3
14	99.9 ± 5.0	n.d	346 ± 14[c]	17.6	82.4
21	153.0 ± 4.0	30.1 ± 3.8	548 ± 13	36.1	63.9
28	157.7 ± 2.7	49.0 ± 2.7	934 ± 117	76.7	23.3
35	163.9 ± 2.5	63.3 ± 2.6	1649 ± 344	88.7	11.3
42	169.7 ± 3.6	100.8 ± 5.8	2790 ± 118	87.8	12.2
49[d]	174.9 ± 5.7	115.4 ± 6.5	2982 ± 173	81.7	18.3
56[e]	118.5 ± 8.3	74.6 ± 6.8	3061 ± 293	78.4	21.6

[a]Data are means ± SE of triplicate determinations on samples from each of 4 field replications.
[b]not determined
[c]nitrogen per seed
[d]Dehiscence had not yet occurred.
[e]Carpels had dehisced in > 50% of the samples.

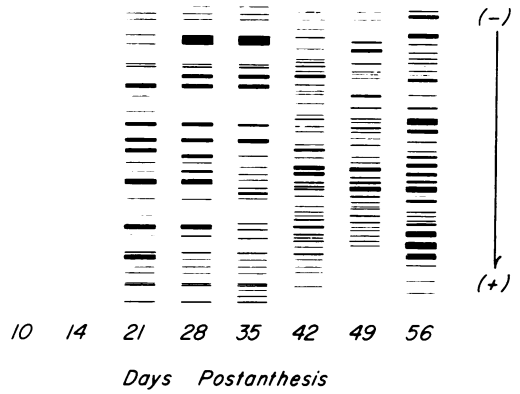

FIG. 2. Diagrammatical representation of protein patterns from gradient electrophoresis of aqueous extracts of cotton embryos from 10 to 56 days postanthesis. No proteins were detected at 10 or 14 days.

date thereafter. There was a relatively large number of intense bands at 56 days (when the seeds were essentially mature).

Following an initial peak at 21 days, the content of water-soluble nitrogen declined until the 6th week, during which it began to increase again (Fig. 1). It reached its maximum value at maturity, although the rate of increase during the final week was low. A majority of the water-soluble nitrogen late in development was probably in the form of peptides. We found 52% of the water-soluble nitrogen, 297 μg/embryo, to be soluble in 10% trichloroacetic acid at 49 days. Elmore and Leffler (6) reported only 35 μg nonprotein nitrogen/seed at a comparable time. Their procedure of extraction with acid-alcohol, followed by passage through Dowex 2-X8 resin, should exclude peptides; these compounds should be soluble in 10% trichloroacetic acid.

Alkali-soluble Nitrogen. During the initial 3 weeks of development, a relatively small amount of nitrogen was extractable with NaOH (Table I and Fig. 1). By early in the 4th week, more nitrogen was extracted with alkali than with water. This is the time during which the crude protein fraction of cotton seeds begins to change qualitatively, indicating synthesis of storage proteins (6). Interestingly, electron microscope studies of cotton (4) indicate that at this time (20–25 days postanthesis) the protein bodies characteristic of oilseed storage proteins are not yet detectable. Instead, membrane-bound electron-dense granules are found associated with Golgi vesicles in the cytoplasm. These granules stain with mercuric bromphenol blue, a protein-specific stain (19), and they are destroyed by pronase (4). Only later in development do the vacuolar storage protein bodies appear that are characteristic of mature cotton seeds.

The changes in quantity of alkali-soluble nitrogen during the early weeks of development were paralleled by changes in quality as measured by SDS electrophoresis (Fig. 3). As implied above, the only detectable proteins at 10 and 14 days were in the NaOH extracts; the electrophoretograms at those times contained minimal numbers of faint bands, quite dissimilar from those characteristic of the later developmental stages.

Concomitant with the quantitative increase in alkali-soluble nitrogen during the 4th week, there was a marked change in its electrophoretic profile (Fig. 3). Most bands increased in intensity and new bands appeared between 21 and 28 days; the 28-day SDS profile was the first to bear a resemblance to that of the mature cotton seed. The SDS electrophoresis pattern of alkali-soluble proteins between 35 days and maturity was characterized mainly by continued intensification of bands present at 28 days. The 56-day profile closely resembled that of storage proteins isolated from mature cotton seeds by a nonaqueous method (15) that relies upon structure rather than solubility for the isolation of proteins (Fig. 4).

The consistent similarity among the SDS profiles of the 28-day through the 56-day alkali-soluble proteins from developing cotton seeds and, in turn, that of isolated storage proteins leads to the conclusions that: (*a*) alkali-soluble proteins of developing cotton seeds include storage proteins; and (*b*) synthesis of storage proteins has therefore been initiated by the 28th day postanthesis. Two of the protein bands present in the 56-day profile (mol wt 52,700 and 45,600) were first observed in the profile at 21 days postanthesis; most of the final SDS profile could be identified by 28 days postanthesis. Similarly, previous amino acid analyses of developing cotton seeds showed the 28-day stage to be the first to resemble mature seed amino acid profiles (6).

There were some changes in the SDS profiles of the alkali-soluble proteins between 28 and 56 days postanthesis, however. Two bands (mol wt >60,000) that initially formed early in development apparently peaked in concentration at 35 days, then diminished; they were last seen at 42 days. Another band (mol wt ~25,000) was transitory and was observed only at 42 days, while a minor component of the mature seed protein profile (mol wt 32,000) was first apparent at 42 days.

Although these changes were relatively minor, it may be significant that they seem to center around the period 42 days postanthesis and seem to involve the diminution of some of the high mol

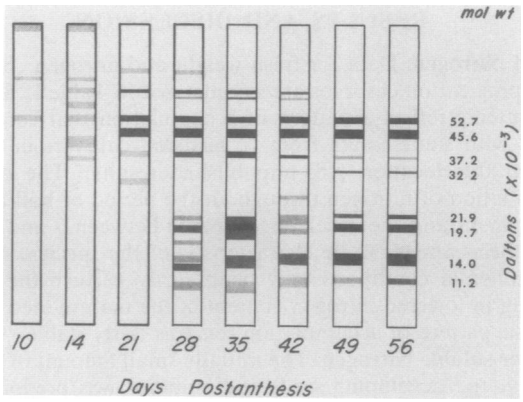

FIG. 3. Diagrammatical representation of SDS electrophoresis patterns of NaOH extracts of cotton embryos from 10 to 56 days postanthesis. Apparent mol wt of polypeptides in 56-day extract are shown at the right. Plotted migration values are means of at least four replicate separations at each date.

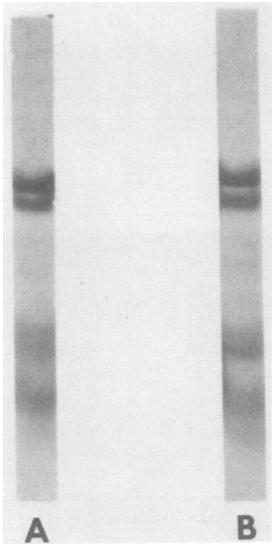

FIG. 4. SDS electrophoretic separation of: (A) alkali-soluble proteins from cotton embryos at 56 days postanthesis, and (B) storage proteins from protein bodies isolated from mature cotton seeds by a nonaqueous method (15).

wt bands and the relative increase of low mol wt proteins. This 42-day postanthesis period is also that during which there were increases in the amount and complexity of the water-soluble proteins. Although Elmore and Leffler (6) reported very low levels of nonprotein nitrogen during the maturation of cotton seed, we found the level of trichloroacetic acid-soluble nitrogen in the water extract of seeds 49 days postanthesis to be relatively high; this water-soluble trichloroacetic acid-soluble nitrogen fraction may include substantial amounts of short peptides. If so, this would indicate a consistent progression within both the alkali-soluble and the water-soluble fractions toward lower mol wt materials. Such a progression would be consistent not only with the pregermination physiology previously suggested by others (5) but also with the apparent decrease in alkali-soluble nitrogen during the latter phase of seed development.

We have shown that two classes of proteins, separable on the basis of solubility properties, accumulate during different periods of cotton seed development. Each of these classes is electrophoretically heterogeneous, which is in agreement with previous reports of research with seed proteins from other species (3, 9–12). Water-soluble proteins predominate during early development, diminish during middle development, then increase again near seed maturity. Alkali-soluble proteins follow a different course during development: they are present in only low amounts during early development, and then increase rapidly during the middle period. On the basis of the time of their synthesis and their electrophoretic similarity to proteins from isolated protein bodies, we believe the alkali-soluble proteins include storage proteins of cotton seed.

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