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Heterogeneity of Catalase in Maturing and Germinated Cotton Seeds[1]

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ABSTRACT

To investigate possible charge and size heterogeneity of catalase (EC 1.11.1.6) in cotton (*Gossypium hirsutum* L. cv Deltapine 62), extracts of cotyledons from different developmental ages were subjected to nondenaturing polyacrylamide gel electrophoresis and isoelectric focusing. Special precautions (*e.g.* fresh homogenates, reducing media) were necessary to prevent artefacts due to enzyme modification during extraction and storage. When the gels were stained for enzyme activity, two distinct electrophoretic forms of catalase were resolved in extracts of maturing and mature cotton seeds. In germinated seeds, three additional cathodic forms were detected revealing a total of five electrophoretic variants. In green cotyledons, the two anodic forms characteristic of ungerminated seeds were less active; whereas, the most cathodic form was predominant. All forms of catalase were found in isolated glyoxysomes. Corresponding electrophoretic patterns were found on Western blots probed with anti-catalase serum; no immunoreactive, catalytically inactive forms were detected. Western blots of sodium dodecyl sulfate-polyacrylamide gels revealed only one immunoreactive (55 kilodaltons) polypeptide in cotton extracts of all developmental ages. Results from isoelectric focusing and Ferguson plots indicate that the electrophoretic variants of catalase are charge isomers with a molecular weight of approximately 230,000.

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) is a principal and characteristic enzyme of peroxisomes (13) and has been continually used as a biochemical and cytochemical marker enzyme for peroxisomes in plant and animal cells (13, 16). Although catalase has been one of the most intensively investigated enzymes, the extent and nature of its multiplicity remains to be resolved. Catalase extracted from tissues and organs of many mammalian species exists as a single electrophoretic form; whereas, multiple electrophoretic forms of the enzyme have been reported in crude extracts of mouse, rat, and rabbit liver (12), human erythrocytes (22), and mouse brain, heart, and spleen (2). Heterogeneity of erythrocyte catalase has been attributed to artefactual oxidation of a single enzyme upon extraction (22); whereas, heterogeneity of catalase in liver appears to be a normal phenomenon occurring *in vivo* (21). It is probable that the multiple forms of liver catalase result from certain epigenetic mechanism(s) acting on the product from a single structural gene (15). In contrast, a true catalase isozyme system, due to the action of at least two genes, has been documented and thoroughly studied in maize (29). Multiple forms of catalase also have been reported for spinach leaves (9), wheat (27), mustard (5), and sunflower (6) seedlings, but in these cases, neither the genetic basis nor physiological function of the isozymes are known.

It has been reported that catalase is sensitive to proteolysis during extraction (3, 16, 20, 23). Mainferme and Wattiaux (20) not only demonstrated that the proteolytic product of catalase had a detectable difference in subunit molecular weight (approximately 2500 D less), but also that it was catalytically active and possessed a different electrophoretic mobility on nondenaturing polyacrylamide gels. Such heterogeneity of an enzyme necessitates extreme caution when looking for a cleavable signal or transit peptide, and when interpreting data on multiple forms of enzymes contained in crude fractions.

As part of our ongoing research on peroxisome (glyoxysome) biogenesis and differentiation in cotton, it was necessary to become aware of any possible catalase heterogeneity, and to determine whether it occurred *in vivo* or was a consequence of extraction procedures. We report here on the *bona fide* existence of multiple forms of glyoxysomal catalase in cotton cotyledons and their differential expression at different developmental ages.

MATERIALS AND METHODS

Chemicals. PMSF,[2] DTT, sucrose, Hepes, 3,3′-diaminobenzidine tetrahydrochloride (grade II), lactalbumin (bovine milk), ovalbumin (grade V), carbonic anhydrase (bovine erythrocytes), BSA (98–99%), catalase (bovine liver; 36,000 units/mg), urease (type IX), alkaline phosphatase conjugated goat antirabbit IgG, fast red violet LB salt, naphthol as biphosphate (sodium salt), benzamidine hydrochloride, leupeptin, and iodoacetamide were obtained from Sigma Chemical Co. Acrylamide, *N,N′*-methylene-bis-acrylamide, bovine serum γ globulin, and mol wt protein standards for SDS-PAGE were obtained from BIO-RAD Lab. Ampholytes (Servalyt 4-7; analytical grade) and SDS (research grade) were obtained from Serva Fine Biochemicals, Heidelberg, FRG.

High quality, deionized H_2O (Barnstead Co., Boston, MA) was used to prepare all aqueous solutions.

Plant Material. Cotton plants, *Gossypium hirsutum* L. $(AD)_1$ cv Deltapine 62 (Delta and Pine Land Co., Lubbock, TX) were grown under the conditions described previously (17), and flowers were tagged at anthesis to determine the age of the developing embryos. For studies with germinated seeds, acid-delinted seeds were surface sterilized in 1% (v/v) NaOCl for 10 min, rinsed thoroughly, soaked in distilled H_2O for 6 h at 30°C with aeration, then scrolled in moistened filter paper. Germination and growth were in the dark at 30°C. All operations with the cotyledons or seedlings (*i.e.* during imbibition, sowing, and harvesting) were performed under a green safelight, although it was determined later that these operations could be done in dim white light without consequence. For studies with green cotyledons, cotton seedlings were grown in moist vermiculite for 10 d in a glasshouse under conditions described previously (17).

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[2] Abbreviations: PMSF, phenylmethylsulfonyl fluoride; dpa, days post anthesis; IEF, isoelectric focusing.

Seeds of *G. klotzschianum* Anderss. (D_{3-K}) and *G. herbaceum* L. (A_1) were provided by R. J. Kohel; seeds of *G. thurberi* Tod. (A_1) were provided by J. R. Radin. Cucumber seeds (*Cucumis sativus* L. cv Improved Long Green) were provided by W. M. Becker. Spinach (*Spinacia oleracea*) was purchased from a local market.

Preparation of Cell-free Extracts. A minimal amount of plant material (*e.g.* one cotyledon pair; 0.15 g), frozen in liquid N_2, was ground to a powder in a cold mortar. Grinding was continued at 4°C with approximately 1 ml of 60 mM Tris-HCl (pH 6.9), 20% (v/v) glycerol, and 1 mM PMSF using a motor-driven Teflon homogenizer (A. H. Thomas Co.). In some experiments (see "Results and Discussion"), the extraction buffer contained 10 mM DTT. The homogenate was centrifuged at 4°C in a microcentrifuge (model 235B, Fisher Scientific) at 13,000*g* for 30 min. The supernatant was used as an enzyme source for nondenaturing PAGE and IEF, and it was applied immediately to the gels. For SDS-PAGE, cell-free extracts were adjusted to 10 mM DTT and 4% (w/v) SDS and immediately placed into a boiling-water bath for 15 min.

Isolation of Glyoxysomes. Glyoxysomes were isolated from dark-grown cotton cotyledons using procedures modified from J. Bradow (personal communication). Cotton cotyledons (approximately 50 pairs) were homogenized with an electric knife in approximately 25 ml of chilled (4°C) grinding medium (pH 7.5) containing 0.4 M sucrose, 50 mM Hepes, 10 mM KCl, 3 mM DTT, 1 mM EDTA, and 1 mM $MgCl_2$. The homogenate was filtered through three layers of Miracloth (Chicopee Mills, Inc.) and centrifuged at 4°C at 250*g* for 10 min. The supernatant was centrifuged at 4°C in a Beckman JS-13 rotor at 19,720*g* for 30 min, and the pellet was resuspended using a camel-hair artist brush in approximately 4 ml of 28% (w/w) sucrose in 10 mM Hepes (pH 7.0). The resuspended organelle pellet was layered onto a sucrose density gradient consisting of 10 ml 60% (w/w) sucrose, 8 ml 40% sucrose, 8 ml 50% sucrose, 8 ml 45% sucrose, 8 ml 40% sucrose, 9 ml 35% sucrose, and 5 ml 30% sucrose. Sucrose solutions contained 10 mM Hepes (pH 7.0). Centrifugation was at 22,000 rpm for 3 h at 4°C using a Beckman SW 25.2 rotor. Two-ml fractions were collected from the top (ISCO model 640 density gradient fractionator, Lincoln, NB). The positions of glyoxysomes and other organelles were determined by sucrose density and profiles of marker enzymes. Glyoxysomes were broken osmotically by diluting the 2-ml fraction (peak catalase activity) with 3 ml of 0.15 M NaCl, 0.10 M Tris-HCl (pH 6.9), 3 mM EDTA, and 2 mM PMSF. For nondenaturing PAGE and IEF, membranes were removed by centrifugation at 100,000*g* for 1 h. For SDS-PAGE, broken glyxoysomes were adjusted to 20% glycerol, 10 mM DTT, and 4% SDS and immediately placed into a boiling-water bath for 15 min.

Preparation of SDS Extracts. Plant material (3 g) was frozen in liquid N_2 and immediately ground to a powder in a cold mortar. The frozen powder was immersed immediately into 20 ml of a vigorously boiling (103°C) solution of 60 mM Tris-HCl (pH 6.9), 20% (v/v) glycerol, 10 mM DTT, and 4% (w/v) SDS for 15 min. The boiled extract was centrifuged at full speed (25°C) in a clinical centrifuge (MSE, model GT-2) for 15 min, and the supernatant was used as an enzyme source for SDS-PAGE and Western blotting.

Catalase Assay and Protein Determination. The catalase reaction mixture contained 3.0 ml of 12.5 mM H_2O_2 in 65 mM K-phosphate (pH 7.2), and 2-5 μl of enzyme solution. The amount of enzyme added was such that the decrease in *A* at 240 nm at 25°C occurred from 0.450 to 0.400 in approximately 60 s; the actual time interval was used for calculation of the first-order rate constant. Protein was determined using Coomassie brilliant blue R-250 (BIO-RAD Lab.) and bovine serum gamma globulin as the protein standard.

Nondenaturing PAGE. Nondenaturing PAGE was performed using the discontinuous buffer system of Davis (4), except the stacking gel and separating gel consisted of 3% polyacrylamide (2:0.5, acrylamide:bis-acrylamide) and 5% polyacrylamide (30:0.8, acrylamide:bis-acrylamide), respectively. Samples (0.01 ml), containing 60 mM Tris-HCl (pH 6.9), 20% glycerol, 0.01% bromophenol blue, and 25 nkat of catalase, were applied to the gel. Electrophoresis was performed at 4°C at 150 V (Mini-Vertical model 360, BIO-RAD Lab.). After electrophoresis, the gels were negatively stained for catalase activity with 3,3′-diaminobenzidine as described by Clare *et al.* (1), or alternatively, the proteins were transferred electrophoretically to nitrocellulose as described below.

For Ferguson plots (7, 10), cell-free extracts (containing 10 mM DTT) from cotyledons of germinated cotton seedlings were electrophoresed in nondenaturing slab gels. Electrophoresis was performed as described above except that (a) 1 mM DTT was included in the gel buffers, (b) the separating gel (15-cm long) consisted of 4.5, 5.0, 5.5, 6.5, 7.0, or 7.5% polyacrylamide, and (c) electrophoresis was performed for approximately 2400 V-h (Protean II, BIO-RAD Lab.) until the tracking dye had migrated to 0.5 cm from the end of the gel.

Commercial preparations of lactalbumin, ovalbumin, carbonic anhydrase, BSA, bovine liver catalase, and urease (5–10 μg each) were electrophoresed simultaneously (in the same gel but different lanes) and stained for protein with 0.12% Coomassie blue.

The R_F of each band was calculated and plots made of $\log(R_F)$ *versus* gel concentration. The slope of each line was obtained from a least-squares linear regression using the SAS computer package (SAS Institute, Cary, NC). From these plots, a standard curve was created where the individual slopes obtained for each standard protein were plotted against the logarithm of their known mol wt (10). From this standard curve, the mol wt of each form of cotton catalase was estimated.

SDS-PAGE. Cell-free extracts, isolated glyoxysomes, or SDS-extracts were subjected to SDS-PAGE using the discontinuous buffer system of Laemmli (19). The staking gel consisted of 4% polyacrylamide (30:0.8, acrylamide:bis-acrylamide), and the separating gel (17 cm long) was 8 or 10% polyacrylamide (30:0.8, acrylamide:bis-acrylamide). After electrophoresis (Protean II, BIO-RAD Lab.), the proteins were transferred electrophoretically to nitrocellulose as described below.

Isoelectric Focusing. IEF was performed in a vertical mini-slab gel (Mini-Vertical model 360, BIO-RAD Lab.) according to the procedures described by Robertson *et al.* (24). The gels consisted of 20% (v/v) glycerol, 5% polyacrylamide (30:1, acrylamide:bis-acrylamide) and 2% (w/v) ampholytes (pH 4–7). Diluted samples (0.01 ml), containing 40% glycerol, 2% ampholytes, and 25 nkat of catalase, were applied to the cathode end of the gel. The anode and cathode solutions were 20 mM acetic acid and 50 mM NaOH, respectively. Electrophoresis was performed at 4°C at 200 V for 2 h followed by 400 V for 2 h.

Electrophoretic Transfer (Western) Blotting. Following electrophoresis, gels were equilibrated for 1 h in transfer buffer (0.192 M glycine, 25 mM Tris, and 20% reagent-grade methanol). Subsequently, proteins were transferred electrophoretically (30 V, 16 h) onto nitrocellulose (0.45 μm; Schleicher & Schuell, Inc., Keene, NH) using a BIO-RAD Trans-Blot cell.

The nitrocellulose blots were soaked for 1 h at 25°C in Blotto (3% Carnation nonfat dry milk, 0.15 M NaCl, 20 mM Tris-HCl [pH 7.8]) as described by Johnson *et al.* (14). Subsequently they were incubated with shaking at 25°C for 2 h in anticatalase serum (18) diluted in Blotto. Control blots were incubated in null (preimmune) serum instead of anticatalase serum. After washing for 1 h in several changes of Blotto, the blots were incubated for 2 h at 25°C with alkaline-phosphatase conjugated goat anti-rabbit IgG also diluted in Blotto. The blots were washed for 1 h in

several changes Blotto, and the immunoreactive catalase polypeptides were localized using a reaction mixture for alkaline phosphatase activity consisting of 0.1 M Tris-HCl (pH 9.2), 2 mM naphthol as-bi phosphate, 1 mM $MgCl_2$, and 0.5 mM fast red violet LB salt (J. Miernyk, personal communication).

RESULTS AND DISCUSSION

SDS-PAGE. Glyoxysomes prepared from dark-grown cotton cotyledons and cell-free extracts of (a) maturing (30 dpa) cotton seeds, (b) dark-grown, and (c) green cotton cotyledons were subjected to SDS-PAGE. The electrophoresed proteins were electroblotted to nitrocellulose, and the blot was probed with monospecific anticotton catalase (Fig. 1). In all samples, a single immunoreactive catalase, with a subunit mol wt of approximately 55 kD, was detected. Hence, the subunit mol wt of catalase is not detectably altered during cottonseed maturation, germination, and postgerminative growth. No larger (59 kD), putative precursor to catalase, similar to that reported on Western blots of germinated pumpkin seedlings (31), could be detected. Similarly, only a single catalase band was obtained when SDS-extracts (see "Materials and Methods"), rather than cell-free extracts, of dark-grown cotton cotyledons were subjected to SDS-PAGE and Western blotting (Fig. 1, lane e).

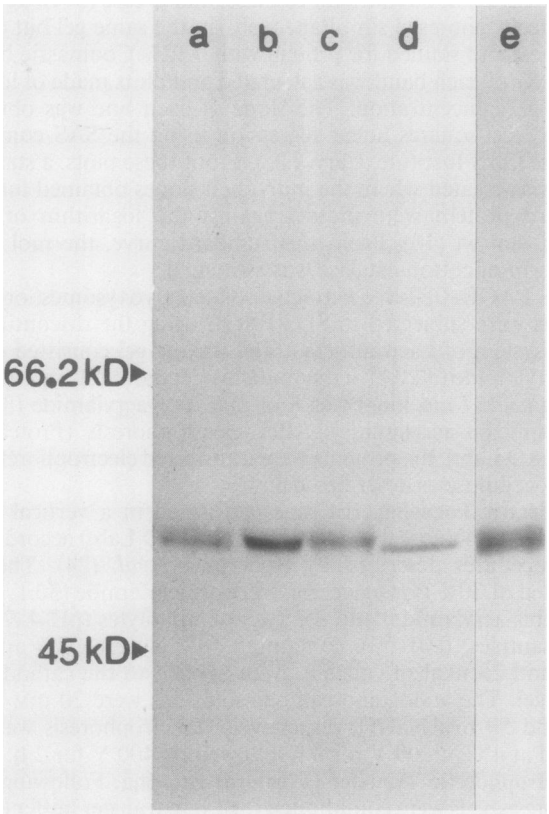

FIG. 1. Comparison of catalase (subunits) in glyoxysomes, cell-free, and SDS extracts of cotton cotyledons using SDS-PAGE and Western blotting. SDS-PAGE was performed in 4% stacking, 8% (17-cm long) separating polyacrylamide gels; electroblots were probed with anticatalase serum. Lanes a, c, d: cell-free extracts (13,000*g* supernatant from cotyledons frozen and powdered in liquid N_2, then homogenized in 60 mM Tris-HCl [pH 6.9], 20% glycerol, 1 mM PMSF) of lane a—maturing cotyledons, c—dark-grown cotyledons, d—green cotyledons. Lane b: isolated glyoxysomes from dark-grown cotyledons. Lanes a to d contained approximately 25 μkat of catalase. Lane e: SDS-extract of dark-grown cotton cotyledons (cotyledons frozen and powdered in liquid N_2, then immediately boiled in 60 mM Tris-HCl [pH 6.9], 20% glycerol, 10 mM DTT, 4% SDS). Lane e contained approximately 100 μg of protein.

Nondenaturing PAGE and IEF. Cell-free extracts of maturing (30 dpa) and mature (50 dpa) cotton seeds were subjected to nondenaturing PAGE. When the gels were negatively stained for catalase activity, two achromatic bands were predominant (Fig. 2A, lanes, a, b). When the electrophoresed proteins were electroblotted to nitrocellulose, and the blots probed with anticatalase serum, two immunoreactive polypeptides also were detected (Fig. 2B, lanes a, b). The immunoreactive polypeptides had the same relative migration as the two catalytically active forms of catalase.

When cell-free extracts of maturing, mature, and desiccated cotton seeds were subjected to nondenaturing IEF, two catalytically active forms of catalase also were observed (Fig. 3, lanes a–c), indicating that the enzymes differ in charge. Unfortunately, when the electrofocused proteins were electroblotted to nitrocellulose using one of several different transfer buffers, catalase could not be detected immunochemically. A possible explanation for this is that catalase precipitates near or at its isoelectric point (26) and cannot easily be electrophoretically transferred from the IEF gel to nitrocellulose.

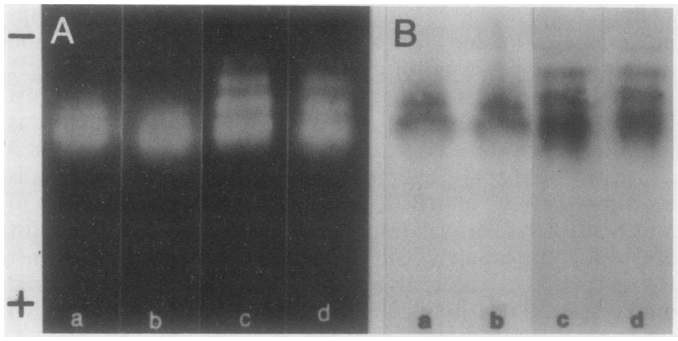

FIG. 2. A, Nondenaturing polyacrylamide (5%) gels showing multiple forms of catalase in cotton cotyledons. Lanes a to c: Cell-free extracts of a—maturing seeds, b—mature seeds, c—dark-grown cotyledons. Lanes d = isolated glyoxysomes from dark-grown cotyledons. All samples contained approximately 25 nkat of catalase and were applied to the gel immediately after preparation. The gels were stained for catalase enzyme activity. B, Western blots, probed with anticatalase serum, of extracts electrophoresed as in A.

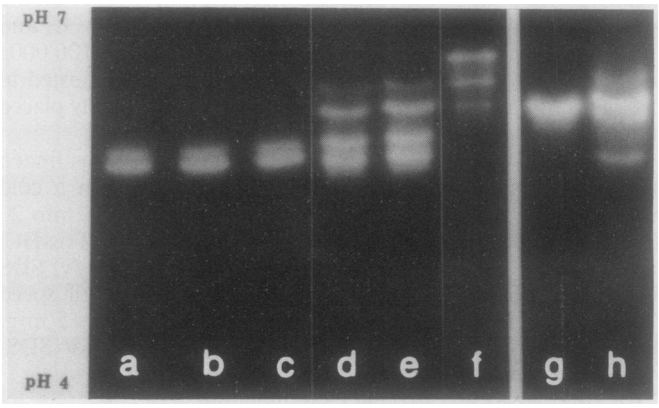

FIG. 3. IEF gels showing multiple forms of catalase in cotton cotyledons (lanes a–f), dark-grown cucumber cotyledons (lane g), and spinach leaves (lane h). Lanes a to d, f: Cell-free extracts of a—maturing cotton seeds, b—mature cotton seeds, c—desiccated cotton seeds, d—dark-grown cotton cotyledons, f—green cotton cotyledons. Lane e: isolated glyoxysomes from dark-grown cotton cotyledons. All lanes had approximately 25 nkat of catalase and were applied to the gel immediately after preparation. All lanes were stained for catalase enzyme activity.

When cell-free and glyoxysomal extracts of cotyledons from dark-grown cotton seedlings were subjected to nondenaturing PAGE or IEF, and the gels negatively stained for catalase activity, one minor (barely visible in these photographs) and four major achromatic bands were observed (Fig. 2A, lanes c, d; Fig. 3, lanes d, e; Figs. 6 and 7). Electroblots of the nondenaturing polyacrylamide gels consistently revealed the four major proteins when probed with anticatalase (Fig. 2B, lanes c, d). Collectively, these results indicate that the two forms of catalase present in immature and mature ungerminated seeds are supplemented during or after germination by three additional cathodic forms of catalase. It appears that heterogeneity of catalase in cotton is analogous to the catalase isozyme system of corn scutellum (25, 29), where catalase isoenzymes characteristic of ungerminated seeds are not replaced by other isozymes following germination but are supplemented by additional forms of glyoxysomal catalase. We previously found using morphometric procedures that peroxisomes of cotton cotyledons persist through seed desiccation and increase dramatically in volume (but not number) during postgerminative growth (17). No evidence was found for autophagy or turnover of entire organelles during the entire period studied (17). The data presented in this study provide additional evidence (albeit circumstantial) favoring the hypothesis that preformed peroxisomes of oilseed cotyledons can acquire enzymes such as catalase posttranslationally without loss of compartmental integrity (17, 28). Direct evidence for the posttranslational acquisition of catalase by preexisting peroxisomes has been reported for rat liver peroxisomes (8).

All five forms of catalase in cotyledons of dark-grown cotton seedlings are present in glyoxysomes (Fig. 2, lane d; Fig. 3, lane 3). Thus, in cotton cotyledons, like the situation in corn scutellum (25) and mustard cotyledons (5), various forms of catalase are not differentially located in various subcellular compartments. This is in contrast with catalase heterogeneity in mouse liver where only the most anodic form of catalase occurs in peroxisomes, and four cathodic forms of catalase are found in the nonsedimentable, soluble fraction (11). To explain the heterogeneity of catalase in mouse liver, it was proposed that one form of catalase, believed to be a glycoprotein, exists in peroxisomes and that the cathodic forms of catalase arise from progressive desialylation following an *in vivo* release of the peroxisomal enzyme into the cytosol (15). In contrast, it has been demonstrated that peroxisomes isolated from rat liver contain all of the various forms of catalase (20), none of which are glycoproteins (30).

A different pattern of catalase multiplicity was revealed when cell-free extracts of green cotton cotyledons were subjected to nondenaturing IEF (Fig. 3, lane f). The most cathodic form of catalase, supplementing the two forms of catalase characteristic of dark-grown seedlings, became more predominant. The two anodic forms of catalase, characteristic of ungerminated seeds, apparently were less active and observed only when gels were relatively overloaded.

It appears that heterogeneity of cotton catalase in greening cotyledons is somewhat similar to catalase heterogeneity reported by Drumm and Schopfer (5) for greening mustard seedlings, where multiple forms of glyoxysomal catalase (in dark-grown cotyledons) are supplemented by additional forms of peroxisomal catalase in a phytochrome-mediated event. Multiple forms of catalase also have been demonstrated in spinach leaves (9), wheat (27), and sunflower seedlings (6). In this study we demonstrate using nondenaturing IEF (Fig. 3, lane h) that catalase heterogeneity in spinach leaves, like the situation in cotton, is due to differences in charge. It is interesting that catalase in cotton, corn, mustard, spinach, sunflower, and wheat is heterogeneous; whereas, catalase in *Lens culinaris* exists in only one major form (26), as it does in cucumber cotyledons (Fig. 3, lane g).

To ascertain whether the electrophoretic variants of cotton catalase observed on native gels were due to differences in size (*e.g.* mol wt isomers), cell-free extracts were subjected to nondenaturing PAGE in slab gels of varying polyacrylamide concentration (7, 10). A plot of $\log(R_F)$ *versus* gel concentration for each of the five catalase variants is shown in Figure 4. Statistical analyses with the SAS computer package revealed that there is no significant difference ($P = 0.79$) among the slopes; whereas, the *y*-intercepts were significantly different ($P = 0.0001$). These data lead to the interpretation that the five forms of cotton catalase are charge isomers with the same molecular size. The mol wt of each of the five forms was determined to be approximately 230,000 (Fig. 5). Similar values, obtained from gel filtration and rate-zonal centrifugation in sucrose, have been reported for catalase from other plant sources (*e.g.* 26, 31), although this study represents the first report of mol wt values for various forms of catalase present in a single organ.

It remains to be determined whether the heterogeneity of cotton (and also mustard, spinach, sunflower, and wheat) catalase

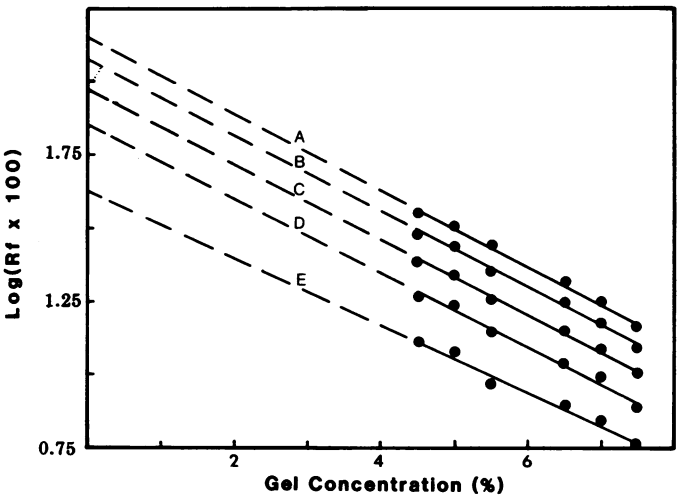

FIG. 4. Mobility of the five forms of catalase from cotyledons of germinated cotton seedlings in nondenaturing gels of differing polyacrylamide concentration. The catalase variants were designated A through E according to their electrophoretic mobility (A is most anodic, E is most cathodic). There is no significant difference among the slopes ($P = 0.79$), whereas the *y*-intercepts are significantly different ($P = 0.0001$).

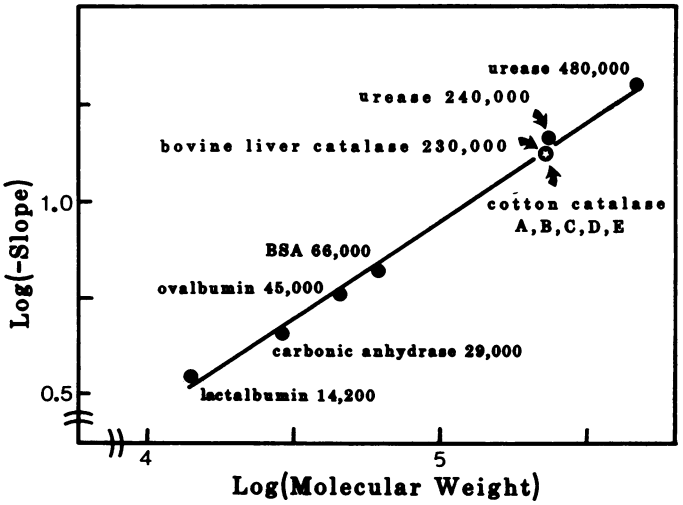

FIG. 5. Estimation of the native mol wt of the five forms of cotton catalase (A through E) determined from data in Figure 4.

is due to the transcription of different genes as shown to be the case for the catalase isozyme system in corn (29), or whether it originates by some epigenetic mechanism. Another possibility, of course, is that multiplicity of catalase is a consequence of genetic variability or homogenization artefacts. These possibilities are discussed below.

In each of our experiments, cell-free extracts were obtained from only one individual. Therefore, the electrophoretic variants of catalase observed represent an individual genotype rather than a population of potentially variable individuals. The electrophoretic patterns in the gels presented in this paper have been reproduced many times, indicating that catalase heterogeneity is not due to allelic variation.

Cultivars of *G. hirsutum* are tetraploid and result from the combination of two diploid genomes designated as A and D. To test whether catalase heterogeneity in *G. hirsutum* is due to polyploidization of the genome, cell-free extracts of dark-grown cotyledons from seedlings of three diploid cotton species (A group—*G. herbaceum*, *G. thurberi*; D group—*G. klotzschianum*) were subjected to nondenaturing IEF. Staining the gels for catalase activity (Fig. 6) revealed the same pattern of multiple electrophoretic variants that is observed in the tetraploid *G. hirsutum* (Fig. 3, lanes d, e).

The electrophoretic pattern of cotton catalase was not altered when the extraction buffer contained 0.31 mM leupeptin (*cf.* 20), 5 mM iodoacetamide, 50 mM EDTA, and/or 1 mM benzamidine hydrochloride (data not shown), indicating that heterogeneity of cotton catalase is not due to proteolysis during extraction. This is substantiated further by the fact that only one catalase band, with a subunit mol wt of 55 kD, was detected on Western blots of SDS gels (Figs. 1 and 7B) containing the same cell-free extracts that were used for nondenaturing PAGE and IEF (Figs. 2–7).

The activity of catalase and electrophoretic pattern from fresh extracts (Fig. 7, lane a) were not altered when 10 mM DTT was included in the extraction buffer to prevent possible alternative oxidation states of a single enzyme (*cf.* 22) or binding of glutathione to free sulfhydryl groups (*cf.* 12). Furthermore, the same electrophoretic patterns were obtained when plant tissue was homogenized under nitrogen gas, with and without EDTA, and when gels were preelectrophoresed (see Ref. 22 for artefacts resulting from oxidizing conditions).

The electrophoretic pattern of cotton catalase in extracts without DTT was modified, however, during storage. When cell-free extracts of cotton were stored at 4°C overnight (16 h), the multiple forms of catalase fused to form one large, more acidic band or smear (Fig. 7A, lane c). This phenomenon was not due to proteolysis during storage because only one band, with a subunit mol wt of 55 kD, was detected on Western blots of SDS gels containing 1-d-old cell-free extracts (Fig. 7B). Inclusion of 10 mM DTT in the extraction buffer preserves the original pattern (Fig. 7A, lane b) and stabilizes catalase enzyme activity for at least 72 h. Extracts without DTT gave distinct bands without extensive smearing only if electrophoresis was performed soon after extraction. This storage phenomenon also has been reported by researchers studying catalase in other laboratories (5, 22). These results demonstrate the need for precautions when preparing extracts for isozyme analysis because, as demonstrated in Figure 7, one could incorrectly interpret the anodic smear as a single electrophoretic band if less catalase was applied to the gel.

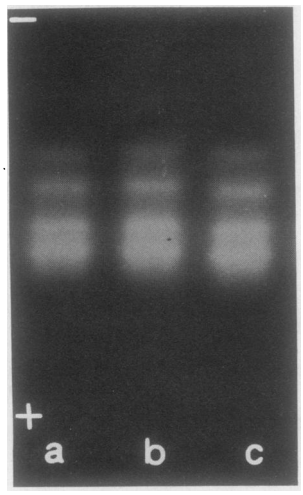

FIG. 6. IEF gel of catalase in three different diploid species of cotton. Lanes a to c: Cell-free extracts of dark-grown cotyledons of a—*G. klotzschianum* Anderss. (D_{3-K}), b—*G. herbaceaum* L. (A_1), and c—*G. thurberi* Tod. (A_1). All lanes were stained for catalase enzyme activity.

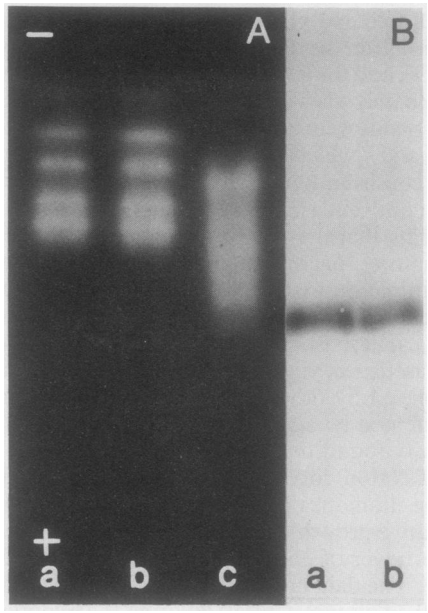

FIG. 7. A, IEF gel of catalase from dark-grown cotyledons of cotton seedlings. Lanes a to c: Cell-free extracts which (a) did not contain DTT, but were applied to the gel immediately after extraction, (b) contained 10 mM DTT and was stored at 4°C for 72 h before being applied to the gel, and (c) did not contain DTT and were stored at 4°C for 24 h before being applied to the gel. Lanes a to c were stained for catalase enzyme activity. B, SDS-PAGE and Western blotting of catalase from dark-grown cotton cotyledons. Lane a: cell-free extract containing 10 mM DTT. Lane b: cell-free extract not containing DTT. Both samples were stored at 4°C for 24 h before being subjected to SDS-PAGE.

From this study, we are certain of the *in vivo* occurrence of multiple forms of catalase in cotton cotyledons. It remains to be determined whether heterogeneity of cotton catalase originates from some posttranscriptional or posttranslational epigenetic mechanism that is differentially active at different developmental ages. An intriguing possibility is that multiple electrophoretic variants are the result of different stages in the biosynthesis, or the turnover, of the enzyme. Alternatively, heterogeneity of cotton catalase could be a true isozyme system in which catalase is controlled by at least two genes. Like the situation in corn (29), it is possible that at developmental ages when more than one gene is expressed, hybrid isozymes of catalase are formed. The appearance of exactly five electrophoretic variants is behavior consistent with randomization of two distinct gene products to form the tetrameric enzyme.

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