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Two temporally synthesized charge subunits interact to form the five isoforms of cottonseed (*Gossypium hirsutum*) catalase

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Five charge isoforms of tetrameric catalase were isolated from cotyledons of germinated cotton (*Gossypium hirsutum* L.) seedlings. Denaturing isoelectric focusing of the individual isoforms in polyacrylamide gels indicated that isoforms A (most anodic) and E (most cathodic) consisted of one subunit of different charge, whereas isoforms B, C and D each consisted of a mixture of these two subunits. Thus the five isoforms apparently were formed through combinations of two subunits in different ratios. Labelling cotyledons *in vivo* with [^{35}S]methionine at three daily intervals in the dark, and translation *in vivo* of polyadenylated RNA isolated from cotyledons at the same ages, revealed synthesis of two different subunits. One of the subunits was synthesized in cotyledons at all ages studied (days 1–3), whereas the other subunit was detected only at days 2 and 3. This differential expression of two catalase subunits helped explain previous results from this laboratory showing that the two anodic forms (A and B) found in maturing seeds were supplemented with three cathodic forms (C–E) after the seeds germinated. These subunit data also helped clarify our new findings that proteins of isoforms A, B and C (most active isoforms) accumulated in cotyledons of plants kept in the dark for 3 days, then gradually disappeared during the next several days, whereas isoforms D and E (least active isoforms) remained in the cells. This shift in isoform pattern occurred whether seedlings were kept in the dark or exposed to continuous light after day 3, although exposure to light enhanced this process. These sequential molecular events were responsible for the characteristic developmental changes (rise and fall) in total catalase activity. We believe that the isoform changeover is physiologically related to the changeover in glyoxysome to leaf-type-peroxisome metabolism.

INTRODUCTION

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) is a characteristic enzyme of peroxisomes in higher-plant cells. During the heterotrophic, post-germinative growth phase of oil-rich seedlings (such as cotton), catalase degrades toxic H_2O_2 derived from β-oxidation of fatty acids within specialized peroxisomes called glyoxysomes. When seedlings assume photoautotrophic metabolism, H_2O_2 produced from oxidation of glycolate in the photorespiration pathway is decomposed by catalase within leaf-type peroxisomes, which are derived from glyoxysomes [1,2]. The enzyme was one of the first enzymes to be isolated in high purity. It has been extensively studied from various plant [3–8] and animal [9–11] sources and generally is a tetrameric, haem-containing protein with a native M_r of 220000–240000. Catalase and most peroxisomal enzymes are synthesized on polysomes in cytosol [2,6,10,12,13], but biogenesis of catalase is not well understood in terms of post-translational modifications of multiplicity. Multiple forms of the enzyme have been reported for many higher plants, e.g. spinach (*Spinacia oleracea*) [3], wheat (*Triticum aestivum*) [14], mustard (*Sinapsis alba*) [15], maize (*Zea mays*) [4,5], sunflower (*Helianthus annuus*) [7,16], tobacco (*Nicotiana tabacum*) [17], and cotton (*Gossypium hirsutum*) [18]. Maize is the only source in which its catalase isoenzyme system has been thoroughly studied [4,5].

Kunce *et al.* [6,18] reported that there were five variants of cotton catalase. They were shown to be charge isomers with the same native and subunit M_r (230000 and 57000 respectively). Developmental changes of the enzyme were discovered wherein two anodic catalase forms in maturing and mature seeds were supplemented with three additional cathodic forms after seed germination. However, it was not determined whether the developmental changes of catalase were due to transcription of different genes, as shown for catalase isoenzymes in maize seedlings [4,5], or due to post-translational modification(s). Kunce *et al.* [6] provided evidence that the enzyme underwent no detectable covalent modifications during its uptake into peroxisomes and its subsequent maturation to a tetrameric haem-containing protein. In the present study we employed several electrophoretic techniques combined with translation experiments *in vivo* and *in vitro* to examine the possibilities that isoforms of cotton catalase were composed of two subunits with different charges, and that their biogenesis is controlled by two different genes. The results allowed us to provide a possible explanation, at the molecular level, for the differential expression of cottonseed catalase in cotyledons during post-germinative seedling growth.

MATERIALS AND METHODS

Chemicals

DEAE DE-52 Servacel and ampholytes (Servalyt 3–10, 4–9, 5–7 and 6–7) were purchased from Serva Fine Biochemicals, Westbury, NY, U.S.A. Urea (ultra-pure) and $(NH_4)_2SO_4$ (ultra-pure) were obtained from Schwarz/Mann, Cambridge, MA, U.S.A. L-[^{35}S]Methionine (1000–1300 Ci/mmol; 1 mCi/0.1 ml of 50 mM-Tricine, pH 7.4) was obtained from NEN/du Pont, Boston, MA, U.S.A. Oligo(dT)–cellulose, Sepharose-6B and PD-10 columns were purchased from Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A. Nuclease-treated rabbit reticulocyte-lysate and wheat-germ-extract translation kits were supplied by Promega–Biotech, Madison, WI, U.S.A., nitrocellulose paper was from Schleicher and Schuell, Keene, NH, U.S.A., and 3,3′-diaminobenzidine tetrahydrochloride came from Sigma Chemical Co., St. Louis, MO, U.S.A. All other

Abbreviations used: i.e.f., isoelectric focusing; poly(A)$^+$, polyadenylated; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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materials were obtained as described by Kunce *et al.* [6,18]. Deionized water (Barnstead Co., Boston, MA, U.S.A.) was used to prepare all solutions.

Plant materials

Cotton [*Gossypium hirsutum* L. cv. Deltapine 62 (Delta and Pine Land Co., Lubbock, TX, U.S.A.)] seeds were either rolled in sheets of filter paper ('scrolled') as described in [19] or grown in flat-bottomed pans ('flats') containing vermiculite in a dark chamber at 30 °C for times indicated. In some experiments, dark-grown seedlings were exposed to continuous white light (125 $E \cdot m^{-1} \cdot s^{-1}$) and maintained at 30 °C.

Buffers

Buffer pH values were determined at 25 °C. Homogenizing medium was composed of 50 mM-K_2HPO_4/KH_2PO_4 (KPB) (pH 7.5)/2 mM-EDTA/10 mM-DTT/1 mM-PMSF/1 mM-benzamidine hydrochloride. Buffers used for washing immunoprecipitates were Buffer A [50 mM-KPB (pH 7.5)/150 mM-NaCl/1% (v/v) Triton X-100/2 mM-EDTA/1 mM-PMSF/1 mM-benzamidine hydrochloride/1 mM-iodoacetamide], Buffer B [same as Buffer A, except with 0.1% (v/v) Triton X-100] and Buffer C (same as Buffer A, but without Triton X-100). Transfer buffer for Western blotting was 0.192 M-glycine/25 mM-Tris HCl/20% (v/v) reagent-grade methanol.

Catalase purification

All the catalase used in the present study was at least partially purified. The following purifying scheme was substantially modified from that of Kunce *et al.* [6]; the shorter time involved yielded a much more stable enzyme preparation. Cotyledons were frozen in liquid N_2 and ground to a powder in a pre-chilled mortar. The powder was blended for 1 min at high speed with cold acetone (−20 °C, 4 ml/g of fresh cotyledons), and filtered through one layer of Whatman 1 filter paper with a weak vacuum. Resuspension of the residue in cold acetone and filtration was repeated four times. It was necessary to vacuum-dry the residue to accomplish reproducible $(NH_4)_2SO_4$ fractionation (see below). The dry powder was resuspended in homogenizing medium (3 ml/g of fresh cotyledons) and blended at high speed for 50 s, and centrifuged at 27000 ***g*** for 30 min at 4 °C. The supernatant was fractionated with $(NH_4)_2SO_4$ (27–70% satd. at 4 °C) by adding solid $(NH_4)_2SO_4$ while stirring the mixture. The 70%-satd.-$(NH_4)_2SO_4$ pellet was resuspended in homogenizing medium (0.5 ml/g of fresh cotyledons) and proteins were precipitated with 2.5 vol. of cold 95% ethanol for 2 h at 4 °C while stirring, then centrifuged at 17300 ***g*** for 15 min. The pellet was resuspended in homogenizing medium (0.1 ml/g of fresh cotyledons), and the ethanol-denatured proteins were pelleted at 17300 ***g*** for 15 min. The catalase-enriched supernatant was used for native i.e.f. or for further purification. All the procedures above were done in 1 day.

Residual ethanol must be removed before ion-exchange chromatography; therefore, proteins were re-precipitated by adding $(NH_4)_2SO_4$ to 70% saturation, resuspending pellets in homogenizing medium, and desalting through a PD-10 column equilibrated with 10 mM-Tris/HCl, pH 8.0. The enzyme solution was applied to a DEAE DE-52 column (2.5 cm × 20 cm) equilibrated in 10 mM-Tris/HCl, pH 8.0, washed with same buffer overnight, and catalase was eluted with a linear gradient of 0–0.5 M-NaCl (500 ml total) in 25 mM-Tris/HCl, pH 8.0. Fractions with catalase activity were pooled and concentrated to 1 ml with a Centriprep 30 (Amicon Division, W. R. Grace and Co., Danvers, MA, U.S.A.). This was applied to a Sepharose-6B column (1.5 cm × 100 cm) equilibrated in 50 mM-KPB, pH 7.5. Catalase was eluted with the same buffer overnight, and concentrated as described above. This preparation of catalase was used for native PAGE. Ion-exchange chromatography and gel filtration were done in less than 2 days.

Gel electrophoresis

Non-denaturing PAGE and staining for protein (80 μkat/lane) or catalase activity (5 nkat per lane) were performed as described by Kunce & Trelease [18]. After electrophoresis, the outer lanes were stained for catalase activity to verify the protein bands stained in the inner lanes, which were removed for urea i.e.f. described below.

Non-denaturing i.e.f. and denaturing urea i.e.f./PAGE were done with either a Mighty Small Model SE 200 or Tall Mighty Small Model SE 280 gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.), the procedure of Robertson *et al.* [20] being used. After i.e.f., gels were stained for catalase activity, or proteins were transferred electrophoretically to nitrocellulose paper as described below. In other experiments, one lane was removed and stained for catalase activity, and catalase isoforms in unstained lanes were removed according to the position of isoforms in the activity-stained lane. These gel slices were used for catalase extraction or directly for denaturing urea-i.e.f. Gel slices were soaked in urea-i.e.f. sample solution [8 M-urea/2% (v/v) Servalyt 3–10/10% (v/v) glycerol in water] for 1 h and then inserted into sample wells of the gel. Focusing was performed at 4 °C at 250 V for 2.5 h and 500 V for an additional 3 h. To determine the pI of isoforms, gels were rinsed briefly with deionized water and the pH gradient was measured on the gel surface with a flat-bottomed pH electrode.

Western blotting

After electrophoresis, i.e.f. gels, with or without urea, were rinsed with water, incubated with shaking in 100 mM-Tris/HCl (pH 6.8)/1% (w/v) SDS/10% (v/v) glycerol/1% (v/v) β-mercaptoethanol for 30 min (i.e.f. gels) or 60 min (urea-i.e.f gels). Two buffer changes during the 60 min were necessary to remove urea from the gels to enhance SDS binding to proteins. The gels were then rinsed with transfer buffer, proteins were transferred at 70 V for 5 h (i.e.f. gel) or 80 V overnight (16 h, urea-i.e.f. gel), and blots were stained as described by Kunce & Trelease [18].

RNA isolation and translation *in vitro*

RNA was isolated from 8 g of 24, 48 and 72 h-old cotyledons (from 'scrolled' seedlings) using the guanidine thiocyanate/CsCl extraction procedure of Maniatis *et al.* [21]. Poly(A)$^+$ RNA was separated and collected by oligo (dT)–cellulose chromatography. Cell-free translation of poly(A)$^+$ RNA was performed in a commercial rabbit-reticulocyte-lysate or wheat-germ-extract system with [^{35}S]methionine, the procedure recommended by the supplier being used.

Radiolabelling *in vivo*

[^{35}S]Methionine diluted in water was applied to cotyledons of intact seedlings [6] in the dark for 1 h. Cell-free extracts were prepared as previously described [18].

Immunoprecipitation of catalase

Immunoprecipitation of catalase synthesized *in vitro* was performed according to the third variation described by Kunce *et al.* [6], except that mixtures were diluted with 1 ml Buffer A and incubated in ice/water for 15 min, and 5 μl of anti-(cotton catalase) IgG was added. Catalase labelled *in vivo* was immunoprecipated as described above, except that 14 μl of anti-catalase serum was used, and purified catalase was not added as a carrier. Immunoprecipitated catalase, synthesized either *in vivo*

or *in vitro*, was collected by centrifugation at 13000 *g* for 15 min, followed by washing, three times each, with Buffers A, B and C.

Protein determination and catalase-activity assay

These were done as described in [18], except that initial reaction rates of H_2O_2 decomposition were used for the calculation of catalase activity.

RESULTS

Catalase purification

The purification procedure described here for cottonseed catalase can be accomplished within 3 days. The catalase had a specific activity of approx. 900 μkat/mg of protein, which was slightly higher (100 μkat/mg of protein) than that of the homogeneous cottonseed catalase reported by Kunce *et al.* [6]. All five forms of catalase were recovered in the final preparations, although the relative amounts of the cathodic forms, D and E, were much less than the other forms (Fig. 1, upper panel). No other discernable bands were apparent on these Coomassie Blue-stained gels. A minor band underneath the major catalase band (M_r 57000) was detected by silver staining of an SDS-containing gel (result not shown).

Subunits of catalase

Protein bands were removed from non-denaturing electrophoretic gels and subjected to i.e.f. in 8 M-urea to identify the number of subunits per isoform (Fig. 1). It was necessary to apply gel slices in the acidic buffer and focus toward the cathode. Reversing the direction of i.e.f. resulted in alkaline aggregation of proteins in the upper portion of the gel. Staining with Coomassie Blue revealed only one protein band, comprising isoforms A and E, although the pI of the band associated with each form was slightly different. Two bands varying in staining intensities were separated from isoforms B, C and D. One band had the same pI as that of denatured isoform A (subunit 1), and the other had a pI identical with that of denatured isoform E (subunit 2). The same results were obtained when the catalase polypeptides were electroblotted from urea-i.e.f. gels to nitrocellulose and detected with anti-catalase antibody (Fig. 2). It was apparent that cottonseed catalase was composed of two subunits of slightly different charge. Isoform A was composed of four subunits 1 ($4 \times S1$) and isoform E of four subunits 2 ($4 \times S2$), whereas isoforms B, C and D were hybrids of S1 and S2. The staining intensities of Coomassie Blue and anti-catalase serum strongly suggest the following ratios in the hybrid isoforms: $B = 3 \times S1/1 \times S2$; $C = 2 \times S1/2 \times S2$; $D = 1 \times S1/3 \times S2$.

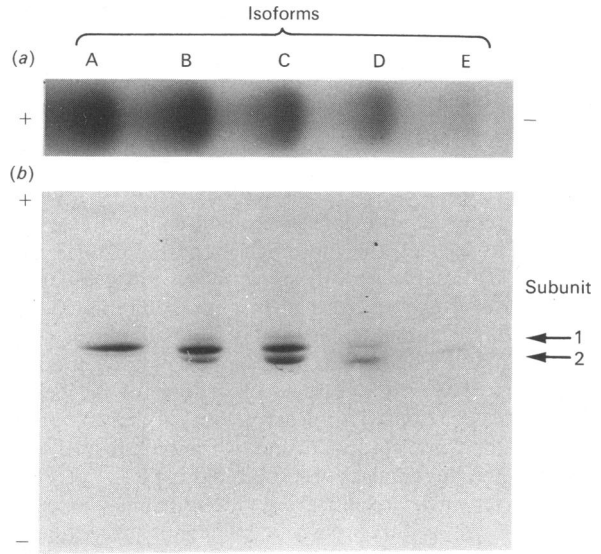

Fig. 1. Non-denaturing PAGE of catalase isoforms and urea i.e.f. of their subunits

(*a*) Five tetrameric isoforms (A–E) of purified catalase (after gel filtration; 80 μkat) separated by non-denaturing PAGE and stained with Coomassie Blue. (*b*) Coomassie Blue-stained subunit(s) (designated 1 or 2) comprising each isoform. A gel slice containing each isoform was removed from a gel similar to that shown in the upper panel, inserted into a well of another gel and subjected to urea i.e.f. (pH 6–7).

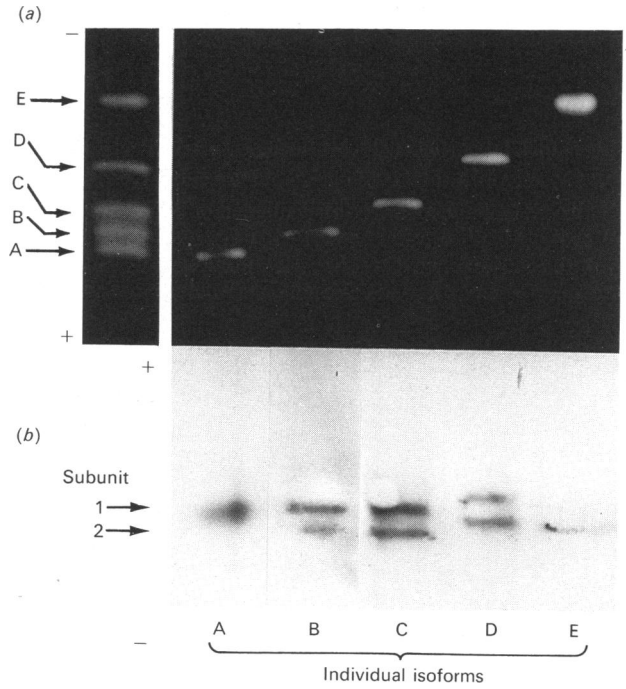

Fig. 2. I.e.f. of catalase isoforms and their subunits

(*a*) Five isoforms (A–E) of partially purified catalase (after ethanol precipitation; 6 nkat) separated by non-denaturing i.e.f. (pH 5–7) and revealed by activity staining (upper left). Protein was extracted from gel slices of each isoform, and added to wells of another i.e.f. gel to show lack of cross-contamination during slicing (upper right), or the entire gel slice was added to separate wells of a gel for urea i.e.f. (pH 6–7). (*b*) Anti-catalase antiserum staining (Western blot) of subunits electroblotted from the urea-i.e.f. gel. Note that the pattern of subunit distribution among isoforms is the same as shown in Fig. 1.

Synthesis *in vivo* and *in vitro* of catalase subunits

[^{35}S]Methionine-labelled cottonseed catalase was extracted and immunoprecipitated from cotyledons of 1-, 2- and 3-day dark-grown seedlings and subjected to urea i.e.f. Coomassie Blue staining of gels (Fig. 3*a*) showed a temporal accumulation of subunits in the cotyledons. Subunit 1 appeared at all three ages and appeared to accumulate as the seedlings aged. Subunit 2 was apparent only at days 2 and 3. The fluorograph from the same gel (Fig. 3*b*) showed which catalase subunit(s) were synthesized within a pair of cotyledons during a 1 h incubation with [^{35}S]methionine. Subunit 1 was a major product in cotyledons at days 1 and 2. The synthesis of subunit 2 was detected only in cotyledons 2 and 3 days old, and it seemed to be

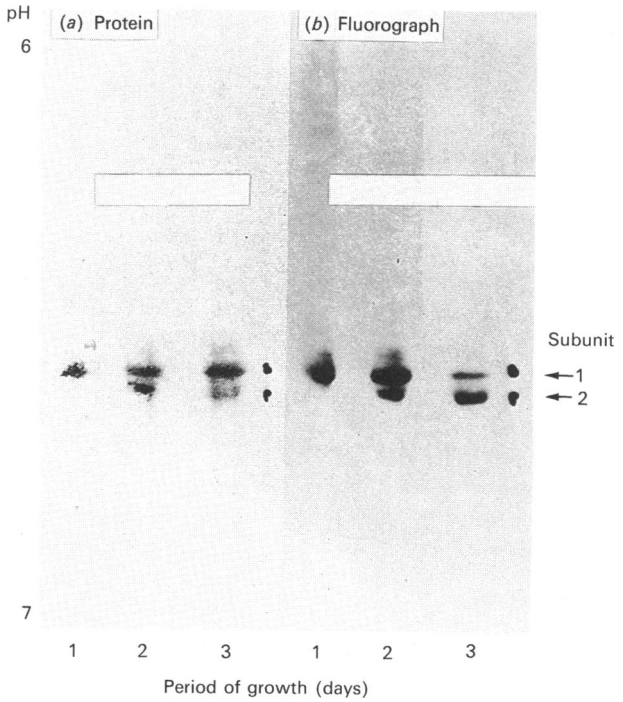

Fig. 3. Synthesis of catalase subunits *in vivo*

(*a*) Coomassie Blue-staining patterns after urea i.e.f. of catalase immunoprecipitated from extracts of 1-, 2- and 3-day-old cotyledons which were incubated for 1 h with [^{35}S]methionine. Catalase from one pair of cotyledons was added to each lane. (*b*) Fluorograph of the urea-i.e.f. gel in (*a*). Radioactive ink spots mark the position of the protein-stained subunits. Notice that the radiolabelling pattern essentially mirrored the protein-staining pattern of subunits at each day, except at day 3, when the synthesis of subunit 2 seemed greater than that of subunit 1.

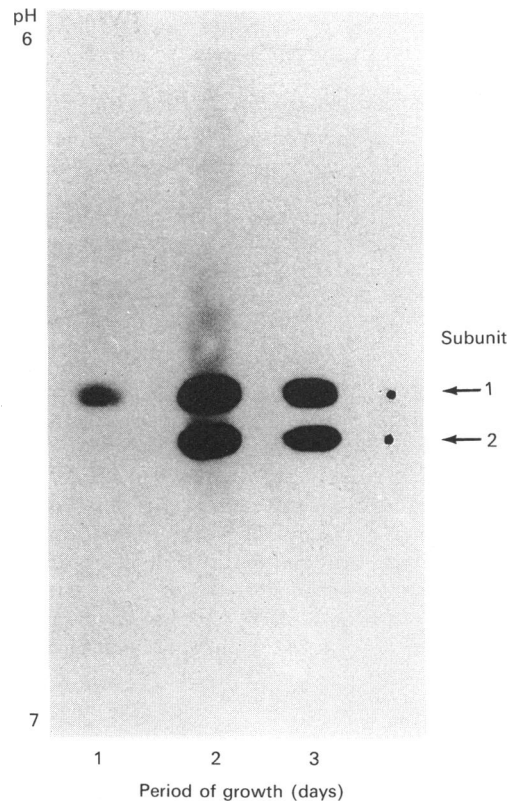

Fig. 4. Synthesis of catalase subunits *in vitro*

Fluorograph of immunoprecipitates from wheat-germ '*in vitro*' translation systems programmed with poly(A)$^+$ RNA extracted from cotyledons of 1-, 2- or 3-day-old dark-grown seedlings. Separations were by urea i.e.f. Ink spots mark the position of subunits immunoprecipitated from 2-day-old cotyledons.

greater than that of subunit 1 at day 3. Synthesis of catalase was not detected when 5-day-old cotyledons (3 days in the dark and 2 days in the light) were incubated for 1 h with [^{35}S]methionine (results not shown). Results similar to those in Fig. 3 were obtained when poly(A)$^+$ RNA extracted from cotyledons of the same ages was translated *in vitro* (Fig. 4). Subunit 1 was synthesized at all three ages, whereas subunit 2 was detected only in immunoprecipitates from RNA extracted at day 2 and day 3. This differential expression of catalase subunits resulted in the differential expression of the tetrameric isoforms (Fig. 5). The two acidic isoforms (A and B) occurring in imbibed seeds were temporally supplemented with three basic isoforms (C, D and E).

Developmental change of catalase

The total catalase activity in cotton cotyledons rose from a low level in dry seeds, reached a peak between 2 and 3 days after soaking, and then declined over the next 2 days, whether the seedlings were grown in vermiculite or in paper scrolls (Fig. 6). The decline was similar whether the seedlings were kept in the dark or exposed to light. Accumulation of isoforms A and B and the appearance of isoforms C and D (Fig. 5) occurred during the period of the most rapid increase in total catalase activity (days 1–2). The amount of isoform A, B and C protein decreased when total activity in the cotyledons was decreasing, indicating that isoforms A, B and C have higher specific activities than those of isoforms D and E. It appeared that exposure of seedlings to light accelerated the appearance of the basic isoforms, but was not required for the production of these isoforms.

pI values of catalase isoforms

Native i.e.f./PAGE was employed for the determination of the pI values of the catalase isoforms (Fig. 7). The three hybrid isoforms had intermediate pI values. The differences between the pI values of neighbouring isoforms were constant.

DISCUSSION

Results reported previously from our laboratory [6,18] stimulated us to seek the molecular basis for catalase heterogeneity. A reasonable hypothesis was that the five cottonseed tetrameric isoforms were formed through combinations of two different charge subunits. Separation of subunits from each isoform required a highly purified, stable catalase containing all the isoforms. The only published procedure for purification of cotton catalase required at least 1 week to accomplish and resulted in the complete loss of the two anodic forms, D and E [6]. Also, when this catalase was subjected to i.e.f., isoforms were partially inactivated and smearing among bands was commonplace. The procedure described herein overcame these shortcomings, yielding catalase which was satisfactory for testing our hypotheses.

Separation and identification of isoform subunits ultimately was accomplished by acid-loading the samples, focusing within a narrow pH (6–7) range, and detecting blotted polypeptides with monospecific antibodies. To our knowledge, this is the first study wherein these techniques and conditions have been applied for examining the subunit composition of catalase isoforms. When catalase, either immunoprecipitated or stored in gel slices, was

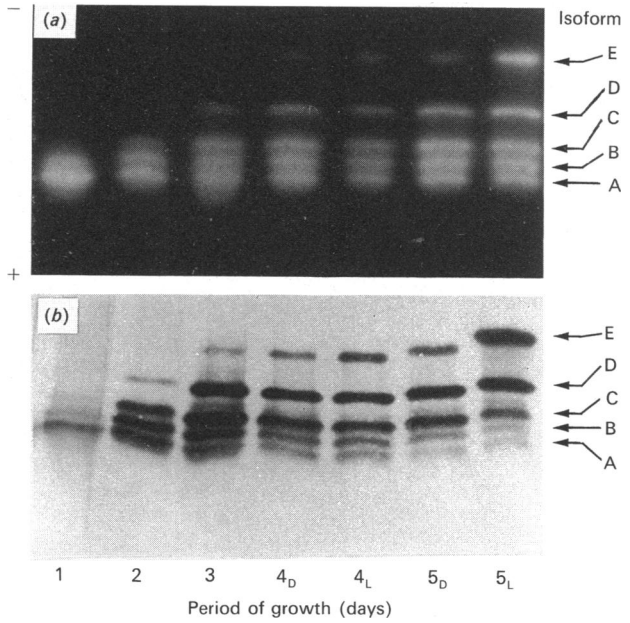

Fig. 5. Comparisons of isoform patterns in light- and dark-grown seedlings

(*a*) I.e.f. gel (pH 5–7) stained for catalase activity (6 nkat/lane). Three active isoforms (C, D and E) sequentially appeared after 2 days growth, whether seedlings were kept in the dark or exposed to light. (*b*) Electroblot of duplicate i.e.f. gel in (*a*), except that 25 nkat of catalase was applied to each lane. Three isoforms (C, D and E) appeared sequentially after day 1, whereas isoforms A and B gradually disappeared. Exposure to light apparently did not affect these trends, but it did appear to enhance the temporal increase in isoform E. The slightly different alignment bands in lanes 1, 2 and 3 and in lanes 5_D and 5_L are not due to difference in migration, but are due to slight distortions that occurred when the 5% gel was placed on the nitrocellulose prior to electroblotting. A similar slight distortion of bands is also apparent in Fig. 2 among lanes C, D and E.

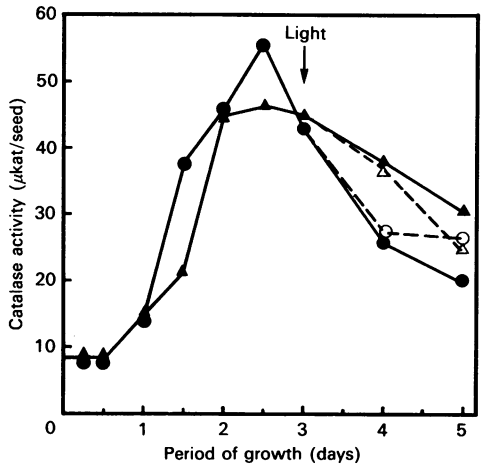

Fig. 6. Time course of catalase activity

Catalase activities were compared for cotyledons of seedlings grown in vermiculite (in flats, ▲) or rolled in sheets of filter paper ('scrolled', ●). Activities were determined in crude extracts prepared from a minimum of 10 cotyledon pairs; the data represent values from at least three replicates. Exposure of seedlings to light (----) had little effect on the decline in catalase activity.

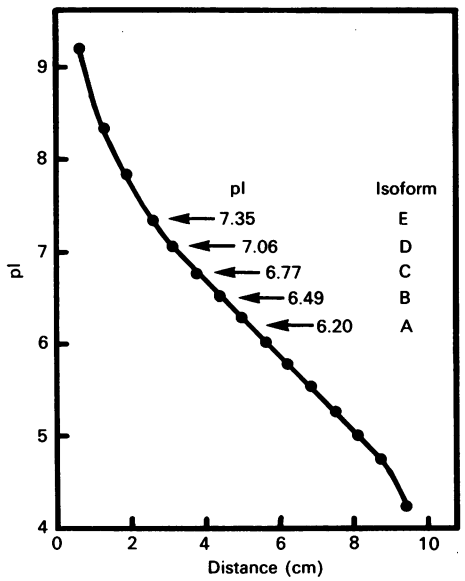

Fig. 7. Isoelectric points of catalase isoforms

Isoforms of partially purified catalase (about 5 nkat) from 4-day-dark-grown seedlings were separated in a non-denaturing i.e.f. gel containing Servalyte 5–7. Focusing was performed for an extra 2 h (7 h total) at 500 V to ensure that all isoforms reached gel regions corresponding to their pI values, which was not the case for the experiments shown in Figs. 2 and 5. Note that the difference between the pI values of neighbouring isoforms is constant.

applied in wells filled with alkaline (~ pH 10) tank buffer, the enzyme aggregated before and/or during focusing. Reversing tank buffers and direction of focusing solved this perplexing problem. A post-electrophoretic washing in a SDS-containing buffer is commonly employed for electroblotting proteins which were not electrophoresed in SDS. However, a procedure for washing urea-i.e.f. gels had not been published, and standard procedures proved unsuccessful. Incubation of these gels for longer time (60 min versus 30 min), including at least two changes of the buffer, was required for satisfactory electroblotting. Microgram quantities of proteins were still required for blotting under these conditions. Urea interfered with SDS binding to proteins; therefore removal of residual urea by extensive washing in SDS probably enhanced the electroblotting process. Catalase commonly consists of four identical subunits. The molecular basis for heterogeneity of the enzyme from different sources [3,6,14–18] (except maize catalase [4,5] and to a less extent that of sunflower [7]), is still unclear. After perfecting our urea-i.e.f. system, we were able to show that tetrameric isoforms of cottonseed catalase were composed of either one or two different charge subunits. According to our hypothesis, hybridization of these subunits should give rise to five tetrameric isoforms with a constant difference in pI between neighbouring forms; the latter was shown to be the case (Fig. 7). It was not known, however, whether the assembly of tetrameric catalase from the two subunits occurred randomly or in a controlled order. Products of the catalase genes *Cat 1* and *Cat 2* in maize were disassociated and rehybridized *in vitro* to form five tetrameric isoenzymes [23]. Similar experiments with cotton isoforms (A and E) yielded only the same two products (active tetrameric A and E; results not shown). Although the results were clearly different, sufficient experimental variations have not been attempted for us to conclude that the assembly of cotton catalase is different from maize catalase *in vivo*.

The factors contributing to charge difference between the two subunits of cottonseed catalase are not known, but several lines of evidence indicate they likely are not derived from post-translational modifications. First, Kunce *et al.* [6] did not find evidence for glycosylation or phosphorylation *in vivo* of the

enzyme, or binding of loosely bound proteins or small molecules. Secondly, they found that catalase, either synthesized *in vivo* or translated *in vitro*, exhibited identical subunit M_r on high-resolution SDS-containing gels. Finally, the two subunits synthesized *in vivo* were indistinguishable in terms of charge from those synthesized *in vitro* (Figs. 3 and 4). Translation *in vitro* of poly(A)$^+$ RNA isolated from cotton cotyledons showed that the two subunits were encoded by two poly(A)$^+$ RNA species (Fig. 4). These two RNA species possibly were transcribed from two catalase genes, or they could have been derived from post-transcriptional modifications (e.g. RNA splicing). To distinguish between these two possibilities, we need to compare cDNA sequences of catalase clones selected from libraries made from poly(A)$^+$ RNA extracted from seeds of different ages.

The time course of total catalase activity in cotton cotyledons (Fig. 6) can be explained by the following sequential molecular events. Sequential synthesis of subunits 1 and 2 led to the accumulation of isoforms A, B and C (the more active forms, Fig. 5); this was followed by selective degradation of these forms. Eising *et al.* [24,25] reported that the decline of total catalase activity in greening sunflower cotyledons was caused primarily by a decrease in the specific activity of the enzyme. Their observations fit quite well with our results in that the rapid decrease of total catalase activity in cotton cotyledons was accompanied by the formation of less active forms of catalase (D and E). Yamaguchi *et al.* [26–28] reported that the temporal decline of catalase in pumpkin (*Curcurbita* sp.) cotyledons was due to a slower processing of the less active form (M_r 59000) to the active form (M_r 55000). Thus the post-germinative changes (rise and fall) of total catalase activity in these three oilseed species seemed to involve post-translational regulation which was manifested by different mechanisms.

Exposure of plants to light was reported to influence catalase expression in leaves of maize [29], and in cotyledons of mustard [15], sunflower [25] and pumpkin [26]. Expression of maize *Cat 2* catalase, an isoenzyme essentially equivalent to cotton catalase isoform E, was shown to be translationally regulated in leaves (not the scutellum) [30]. In mustard, Drumm & Shopfer [15] showed that light, via phytochrome, induced (enhanced?) the appearance of at least seven additional catalase isoenzymes, and that all 12 isoenzymes were present in 18-day-old, white-light-irradiated cotyledons. Exposure of cotton seedlings to light, however, did not appear to be required for the changeover of isoforms from A to E (i.e. the exclusive synthesis of subunit 2), though it seemed to accelerate the process. Also, light exposure had only a subtle, if any, effect on the decline of total cottonseed catalase activity, and isoforms A and B were completely lost in 10-day-old green cotyledons [18]. In spite of these differences, the sequential expression of catalase isoforms appeared to be pre-programmed, i.e. in all cases studied light only acted as a stimulus to enhance the expression of those catalase isoforms serving specific physiological function(s) in leaf-type peroxisomes.

It was apparent that the transition from a predominance of isoform A to isoform E was in concert with the conversion of glyoxysomes to leaf-type peroxisomes. A similar correlative pattern shift also occurred in cotyledons of other epigeal dicotyledonous seedlings (mustard, pumpkin and sunflower) and in the scutellum of hypogeal maize seedlings. Unfortunately, sufficient comparative data are not available to justify conclusion(s) stating that the pattern shift in isoforms is physiologically related to the change in the specific metabolic pathways occurring in glyoxysomes and leaf-type peroxisomes.

In conclusion, cottonseed catalase is composed of two different charge subunits and they are encoded by two poly(A)$^+$ RNA species. The two subunits are differentially expressed during post-germinative growth, resulting in a changeover of catalase isoforms from more acidic forms (A and B) to more basic forms (C, D and E) either in darkness or in the light. Exposure of seedling to light apparently enhances expression of these basic forms, but is not essential. Specific data on properties of the individual isoforms are needed before conclusions can be drawn on the physiological significance of the isoform changeover in cotton and other oil seedlings.

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