Regulation of Maize Catalase by Changing Rates of Synthesis and Degradation

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Rates of catalase synthesis and degradation were measured in the scutellum of the germinating maize seedling by the technique of Price, Sterling, Tarantola, Hartley & Rechigl [*J. Biol. Chem.* (1963) **237**, 3468–3475] by using the porphyrinogenic drug 2-allyl-2-isopropylacetamide to inhibit catalase synthesis. Results indicate that developmental changes in catalase activity are determined by changes in the rate of enzyme degradation as well as by changes in the rates of its synthesis.

During early germination in maize (*Zea mays* L.) scutellar catalase ($\text{H}_2\text{O}_2\text{--H}_2\text{O}_2$ oxidoreductase, EC 1.11.1.6) increases from moderate activity immediately after imbibition to a peak at 4 days of germination, and gradually declines thereafter. These quantitative changes are accompanied by a shift in the isoenzyme complement of the scutellum. While one isoenzyme, encoded by the Ct_1 structural locus, gradually disappears, an isoenzyme determined by a second structural locus (Ct_2) gradually appears (Scandalios, 1975). It has been shown by density-labelling experiments (Quail & Scandalios, 1971) that both isoenzymes are turning over during this period, one as it appears and the other as it disappears. In the present studies, actual rates of catalase synthesis and degradation were measured by the method of Price *et al.* (1963).

Experimental

Germination of seedlings

Maize seeds used in the present studies were all from the highly inbred line W64A. They were soaked in water for 24 h, surface sterilized in 0.5% sodium hypochlorite, planted on moist germination papers in covered trays, and left to germinate in the dark at 25°C; 4-day-old seedlings had a coleoptile length of 3–3.5 cm.

Treatment with 2-allyl-2-isopropylacetamide

Batches of 40 seeds were removed from the germination trays at 24 h intervals for the first 7 days of germination; the scutella were excised and removed

from the embryo. Then 20 isolated scutella were incubated in 5 ml of 0.04 M 2-allyl-2-isopropylacetamide (K & K Laboratories, Plainview, NY, U.S.A.) in 0.2 M sodium acetate buffer, pH 5.6, for 24 h; 15 scutella were incubated in acetate buffer without allylisopropylacetamide as a control. Both incubation mixtures were supplemented with 100 units of penicillin/ml and 20 µg of mycostatin/ml to prevent microbial contamination. The low pH was used to optimize allylisopropylacetamide penetration. Penetration was not enhanced by vacuum infiltration or the inclusion of Triton X-100 in the medium. Five scutella were homogenized as described below and were assayed polarographically for catalase activity (Scandalios *et al.*, 1972). The control and treated scutella were placed on a reciprocating shaker at 25°C. Sets of five scutella were removed from each at 6 h intervals during the subsequent 24 h period, blotted dry and weighed. Each set of five was then homogenized in 5 ml of cold 0.025 M-glycylglycine/HCl buffer, pH 7.4, and the extracts were centrifuged at 27000g for 10 min. The supernatants were decanted and assayed for catalase as described above. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

Determination of rates of synthesis and degradation

Since catalase activities in the scutellum are not constant during the experimental period in question (see Fig. 1), net synthesis could be determined directly by the increase or decrease in enzyme activity during a given 24 h period. Degradation was determined by measuring the decrease in enzyme activity after blocking its synthesis with allylisopropylacetamide. Total synthesis (k_s) was determined for each period by adding the net synthesis and the

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degradation. First-order degradation constants (k_D) were calculated by dividing the catalase activity (units) degraded during allylisopropylacetamide treatment by the sum of the catalase activity at the beginning of the period and the total activity synthesized during the period. Half-lives of the enzymes were determined by dividing 0.693 (which is $\ln 2$) by k_D (Rechcigl, 1971).

Pulse-chase labelling of scutellar proteins

At the beginning of each 24h period, 20 scutella were isolated from seeds germinated as described above, and were incubated in 10ml of 0.2M-sodium acetate buffer, pH 5.6, containing 5 μ Ci of DL-[14 C]leucine (Amersham-Searle, Des Plaines, IL, U.S.A., 60mCi/mmol), 150 units of penicillin/ml, and 0.04M-allylisopropylacetamide. After 6h, the label was chased by washing the scutella twice with acetate buffer and incubating in allylisopropylacetamide solution containing 10mM unlabelled leucine. Three scutella were then removed at 4h intervals and homogenized in 3ml of 0.025M-glycylglycine/HCl buffer, pH 7.4. Extracts were centrifuged at 8000g for 45min, 10ml of cold 10% (w/v) trichloroacetic acid was added to 0.2ml samples of the resulting supernatants, and the precipitates were stored overnight at 4°C. Precipitated proteins were then collected on single glass-fibre filters (Gelman, type E), and were washed with 2 $\times$ 5ml volumes of cold 10% trichloroacetic acid. Radioactivity on the filters was then counted in 10ml of a xylene-based scintillation fluid (Multisol; Isolab Inc., Akron, OH, U.S.A.) for 10min in a refrigerated Packard Tri-Carb liquid-scintillation counter.

Effectiveness of allylisopropylacetamide in blocking synthesis

The extent to which allylisopropylacetamide inhibited catalase synthesis was determined by incubating sets of five scutella excised from 3-day-old seedlings in 10ml of the acetate buffer supplemented with penicillin and with or without 0.04M-allylisopropylacetamide. After 7h, 10 μ Ci of [14 C]leucine was added to each flask. Then 17h after the label was introduced, the scutella were removed, homogenized in 2.5ml of water and centrifuged at 27000g for 30min. The supernatant fractions were mixed with an equal volume of anti-catalase serum, the immunological precipitates were collected on micropore filters and the radioactivity was counted as described above. For antibody preparation, the catalase from 3-day-old seedlings was purified to electrophoretic homogeneity by $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel-filtration chromatography and ion-exchange chromatography on DEAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Albino rabbits were

injected intramuscularly with 1mg of the purified catalase each week for 4 weeks and with 2mg each week for the final 3 weeks. The serum was collected via cardiac puncture 1 week after the final injection. Processed sera were stored at -20°C.

Results

The quantitative profile of catalase expression in isolated scutella is shown in Fig. 1. Catalase activity increases during the first few days of seedling development, then decreases after day 4. It has been shown previously (Quail & Scandalios, 1971) that this increase is accompanied by the expression of a second structural gene, and the present studies were initiated to establish the extent to which this new expression determines the developmental profile of catalase activity.

The results of these studies are shown in Table 1. The rates of catalase synthesis increase to nearly 3 times the original value by day 3 of germination. After day 4, rates of catalase synthesis decrease to values approaching those observed during day 1 of germination.

As shown in Fig. 2, catalase decay after inhibition of its synthesis with allylisopropylacetamide follows approximate exponential-decay kinetics, indicating that it is a first-order phenomenon. The deviations from exponential linearity are probably due to the fact that inhibition of synthesis is incomplete (see below). The degradation data are listed in Table 1 in the form of first-order degradation constants, k_D . The striking feature of these values is the variation observed, particularly at days 2 and 3. This indicates that catalase degradation in this system is not at a steady rate, and that changing rates of catalase degradation play a major role in the developmental expression of catalase in this tissue (Fig. 1).

Fig. 1. *Developmental profile of catalase activity in the scutellum during early germination in maize*

A unit of catalase activity is defined as that amount of enzyme causing the liberation of 1 μ mol of O_2 /min under standard conditions. See the Experimental section for details.

Table 1. *Synthesis and degradation of maize catalases during early germination*

All values, except k_D , are expressed in catalase units/mg fresh wt. of scutellum. Degradation and synthesis values are listed as catalase units synthesized or degraded/mg of tissue per day. Calculations are described in the Experimental section. S.D. values are based on a total of seven to twelve replicates in three or four independent experiments.

Period of germination (days)	Control (no allylisopropyl-acetamide)	Net synthesis	Degradation	Synthesis	k_D (unit/day per unit)
0	43.7 ± 7.3	—	—	—	—
1	90.7 ± 9.2	47.0	41.4 ± 4.4	88.4 ± 5.6	0.23
2	181.0 ± 7.7	90.3	39.3 ± 21.6	129.6 ± 11.5	0.13
3	363.9 ± 14.6	182.9	24.0 ± 3.3	206.9 ± 34.0	0.04
4	440.7 ± 19.3	76.8	127.2 ± 2.9	204.0 ± 34.2	0.20
5	410.5 ± 12.5	−30.2	146.5 ± 28.6	116.3 ± 12.1	0.28
6	371.2 ± 16.3	−39.3	118.6 ± 6.3	79.3 ± 14.3	0.26
7	330.3 ± 18.9	−40.9	126.1 ± 23.4	85.2 ± 13.2	0.30

Fig. 2. Decay kinetics of catalase activity in 3-day-old scutella after blocking catalase synthesis with allylisopropylacetamide

These data are representative of the patterns observed during each developmental stage investigated.

Changes in the k_D values could be caused by either changes in the efficiency of the catalase-degrading mechanism (proteinases) or in the susceptibility of the catalase molecules themselves to degradation.

Fig. 3. Half-lives of catalase (▲) and general scutellar proteins (Δ) during early germination

Catalase half-lives were determined with the allylisopropylacetamide technique, and general protein half-lives were determined by pulse-chase labelling with [^{14}C]leucine.

Pulse-chase radiolabelling studies with [^{14}C]leucine were conducted in an attempt to observe any changes in general scutellar protein turnover, since such changes might support the former explanation. As shown by Δ in Fig. 3, no significant changes were, in fact, observed. The catalase half-lives (a function of the first-order degradation constants, k_D) are represented by ▲ in Fig. 3 for comparison.

Discussion

These studies show that the quantitative profile of catalase activity in the maize scutellum during early

germination is determined by changing rates of both synthesis and degradation. The increase in enzyme synthesis before day 4 can be partially attributed to the new expression of an additional structural gene (Ct_2) (Scandalios, 1975). The magnitude of the increase (nearly 3-fold) indicates, however, that either the newly expressed gene has a synthetic rate approximately twice that of the previously expressed gene (Ct_1), or that the synthetic efficiency of both genes increases during this period. The density-labelling studies of Quail & Scandalios (1971) indicated that the two structural genes show approximately a 2-fold difference in their rates of catalase synthesis and support the former explanation. The possibility of translational controls increasing general catalase synthesis has not been rigorously eliminated, however. Uenoyama & Ono (1973) have reported factors specifically controlling the rates of catalase translation in rat liver.

Several possible explanations of the decrease in catalase synthesis after day 4 exist, the most likely being that transcription of the catalase genes has been terminated, and residual synthesis represents the activity of a dwindling supply of relatively stable mRNA.

The present studies show that rates of catalase degradation also play a major role in determining the pattern of catalase expression in the scutellum of the germinating maize seed. Rates of degradation change dramatically during the period examined. Changes in the rates of degradation of an enzyme may be due to changes in the degradation mechanism or to changes in the susceptibility of that enzyme to degradation. Present methodology will not allow us to distinguish between these two alternatives; however, we have some evidence for the latter explanation. The marked increase in catalase stability at day 3 (Fig. 3) may reflect the changing patterns of subcellular compartmentation of the enzyme. A significant portion (30–50%) of the catalase in the cell is found in glyoxysomes at days 3 and 4. Studies on glyoxysome ontogeny in maize scutella indicate that these organelles are few in number before days 2 and 3, and decrease sharply in number after day 4 (Scandalios, 1974; C. P. Longo & Longo, 1970; G. P. Longo & Longo, 1970). These patterns were determined by gradient fractionation studies, glyoxysomal enzyme patterns, total glyoxysomal protein-patterns and electron-microscopic examination. If we assume that the compartmentalized catalase is temporarily protected from degradation, the observed increase in apparent enzyme stability would be expected. After days 4 or 5, compartmentation of the catalase is probably not a significant factor, and after this point catalase degradation does not differ substantially from that of other scutellar proteins (Δ , Fig. 3). This suggests that catalase is probably not being degraded by a specific

and unique system. Caution must, however, be exercised when comparing turnover measurements made by different techniques, since different methods may measure different 'turnover pools' (Rechcigl, 1971; Goldberg & Dice, 1974).

We believe the compartmentation phenomenon to be the most likely explanation of the dramatic variation in catalase stability for the following reasons. (1) We were unable to detect similar changes in general scutellar protein turnover. (2) The dramatic increase in catalase stability is coincident with its compartmentation in glyoxysomes, and its return to higher rates of degradation after day 4 is coincident with the disappearance of these organelles. (3) This explanation obviates the necessity for invoking a catalase-specific proteinase which undergoes multifold variations in activity during early germination, or for some modification of the catalase proteins such that they become temporarily resistant to degradation.

The validity of the actual values obtained for synthesis and degradation rates depends on two factors, the completeness with which allylisopropylacetamide blocks catalase synthesis, and the degree to which allylisopropylacetamide perturbs cellular metabolism.

Although our experimental conditions were designed to give optimum inhibition of catalase synthesis (as judged by the rate of persistence of catalase degradation), the inhibition was not complete. Prolonged treatment with allylisopropylacetamide caused catalase activity to fall to a few per cent of the control value, suggesting that catalase synthesis was not completely blocked. Measurements of [^{14}C]leucine incorporation into immunologically recognizable catalase material indicate that the inhibition was only 70–75% complete (Table 2). ^{59}Fe incorporation studies in rat liver (Price *et al.*, 1963) suggested that the inhibition of catalase synthesis by allylisopropylacetamide in that tissue was approx. 90%. Although any residual synthesis would make the rates of degradation appear somewhat lower than actual values, the relative develop-

Table 2. ^{14}C incorporation into immunological precipitates of maize catalase from 3-day scutella

Results are the average of two independent experiments with five scutella per treatment in each experiment. Conditions are as described in the Experimental section.

Treatment	Incorporation		Inhibition (%)
	(c.p.m.)	(% of control)	
Control	14560	100	—
+0.04M-Allylisopropylacetamide	3856	26.7	73.3

mental pattern, and hence our basic conclusions, would remain unchanged.

The extent to which the observed patterns of catalase turnover reflect turnover patterns *in situ* may be influenced by the effects of allylisopropylacetamide administration on general cellular metabolism. This is a problem encountered in most turnover studies, although one would expect the effects to be generally less for a more specific inhibitor such as allylisopropylacetamide than for a general protein-synthesis inhibitor such as cycloheximide. Pulse-chase labelling studies with [^{14}C]leucine showed no substantial differences in general protein turnover between control and allylisopropylacetamide-treated scutella (results not shown).

On the basis of these studies and the evidence from previous studies (Quail & Scandalios, 1971), we conclude that the expression of catalase in the scutellum of the germinating seed is controlled by changing rates both of synthesis and of degradation. Rates of synthesis are determined, at least in part, by the differential expression of two structural genes with different synthetic efficiencies. Rates of catalase degradation change markedly during the first 7 days of germination, and may be determined by changing patterns of compartmentation of the enzyme.

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