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Cat3, a Third Gene Locus Coding for a Tissue-Specific Catalase in Maize: Genetics, Intracellular Location, and Some Biochemical Properties*

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Summary. A new and unique catalase isozyme, CAT-3, has been found in *Zea mays*. It is encoded in the *Cat3* nuclear structural gene which is distinct from the two previously described catalase structural genes, *Cat1* and *Cat2*. The *Cat3* gene is both tissue- and time-dependent in its expression, being expressed primarily in young leaves and in the pericarp of nearly mature kernels. Cell fractionation experiments, utilizing epicotyl (coleoptile + primary leaf) and mesocotyl cells, suggest that CAT-3 is associated with the mitochondria where it may play a role in the alternate oxidase pathway. CAT-3 was purified and characterized with respect to some of its biochemical properties. While CAT-3 differs in some of its properties from CAT-1 and CAT-2, it is similar to these and to other catalases in most respects.

Introduction

We previously reported (Scandalios, 1965; 1975) that maize catalase (E.C. 1.11.1.6.; H_2O_2:H_2O_2 oxidoreductase) is a tetrameric enzyme encoded in at least two structural genes, *Cat1* and *Cat2*. Six alleles, detectable by conventional electrophoretic procedures, have been recovered at the *Cat1* locus (Scandalios, 1968) and three alleles at the *Cat2* locus (Scandalios, unpublished data). We have recently demonstrated that these two loci are unlinked; *Cat1* has been located on chromosome-5 and *Cat2* on chromosome-1 (Roupakias, McMillin and Scandalios, submitted).

The *Cat1* and *Cat2* genes exhibit differences in their temporal expression in the scutellum. Generally, *Cat1* is expressed during kernel development and *Cat2* during early sporophytic development. There is a period during which both genes overlap in their expression and their subunits interact to generate intergenic hybrid molecules (Scandalios, 1975).

In addition to these temporal changes in catalase gene expression, we have recently discovered a number of situations where a particular catalase appears in a given tissue at a given developmental period. One such case is the presence of a unique catalase isozyme, CAT-3, in the shoot of young seedlings and in the pericarp of nearly mature kernels.

In this paper we report on the genetic basis of CAT-3, its intracellular location and some of its biochemical properties.

Materials and Methods

Preparation of Material and Electrophoresis

A number of highly inbred maize strains were employed in these investigations. The strains W59, A186 and M10A55 are homozygous for the "B" electrophoretic variant of CAT-3, whereas the inbreds A195, W64A, Oh51A and 59 are homozygous for the "A" variant.

For subcellular localization studies and biochemical characterization of CAT-3, the inbred line W64A was used, unless otherwise stated.

Plants were grown either in the field, in the greenhouse, or under highly controlled conditions of temperature, light and humidity in growth chambers depending upon the experimental needs.

Seeds were surface sterilized and germinated in darkness under constant temperature and humidity as previously described (Scandalios, 1974). For genetic analyses, the coleoptile tissue was harvested on the fifth day after germination (about 1–3 cm long) from the appropriate F_1, F_2 and reciprocal backcross seedlings. Individual coleoptiles were ground in 1-drop of 0.025M glycylglycine buffer (pH 7.4) and 1–2 mg PVP (polyvinylpyrrolidone) to remove phenolic compounds. The crude extract was then used directly for electrophoresis. Starch gel electrophoresis, using a lithium borate-tris citrate buffer (pH 8.3), and staining for catalase

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isozymes were conducted precisely as previously described (Scandalios, 1969).

Disc polyacrylamide gel electrophoresis (PAGE) was conducted precisely as previously described by us (Felder and Scandalios, 1973).

Preparation of Subcellular Organelles

For preparation of the subcellular organelles, about 10 g of scutella or 6 g of epicotyls, or primary leaves, were isolated from the same population of seedlings (200 seedlings) and minced with a razor blade in 3 to 5 ml of grinding medium modified from the medium used by Briedenbach and Beevers (1967). Sucrose (0.6 M) in Hepes (N-2-hydroxy-ethylpiperazine-N′-2′ ethanesulfonic acid) buffer was substituted for 0.4 M sucrose in Tris buffer and 0.1% insoluble PVP was added to remove phenolic compounds. The homogenate was filtered through three layers of cheesecloth and centrifuged at $705 \times g$ for 5 min. The $705 \times g$ supernatant, designated as the crude homogenate, was then centrifuged for 15 min at $9{,}500 \times g$ and the pellet was washed with a suspension medium. The suspension medium was essentially the same as the grinding medium except that 0.3 M sucrose was used and PVP was omitted. The washed pellet was designated as washed mitochondria (Mw). Continuous or stepped gradients of sucrose (described in the legend) were prepared in 0.01 M Hepes buffer (pH 7.5) containing 10^{-4} M EDTA and 10^{-3} M DTT (DL-dithiothreitol).

Subfractionation of Mitochondria

For subfractionation of mitochondria, washed mitochondria (Mw) or purified mitochondria (Mp) which were collected from sucrose gradients after equilibrium centrifugation were shocked osmotically with distilled water as described (Rich et al., 1976). The mitochondrial fraction collected from the gradient was carefully adjusted to a concentration of 20% sucrose and centrifuged at $9{,}500 \times g$ for 15 min. An aliquot of the mitochondrial pellet was taken as a reference. The rest was suspended in 0.5 ml concentrated glycerol and stored at 4° C overnight. This glycerol mixture was then diluted, vigorously mixed with 8 ml distilled water, and centrifuged at $9{,}500 \times g$ for 15 min to remove the intact mitochondria. The supernatant was centrifuged at $102{,}900 \times g$ for 60 min. The pellet (sM) was suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3% Triton X-100.

Electron Microscopy

The mitochondrial fractions were pooled and pelleted from sucrose gradients as described above. The mitochondrial pellet was fixed with 0.1 M phosphate buffer (pH 7.4) containing 3% glutaraldehyde and 10% sucrose for 2 hours. Prior to fixation with 2% OsO_4 in 0.1 M phosphate buffer, the mitochondrial pellet was washed with 0.1 M phosphate buffer containing 15% sucrose at 4° C overnight. Dehydration was performed using a graded ethyl alcohol series and embedment in Epon 812 was prepared according to Luft (1961). The sections were stained in 2% aqueous uranyl acetate for 10 min followed by lead citrate for 10 min and viewed in a Hitachi HS-8B electron microscope.

Analytical Methods

Catalase activity was determined essentially as described by Chance and Maehly (1955), isocitrate lyase according to Dixon and Kornberg (1959), and cytochrome oxidase according to Smith (1955). Reduced cytochrome c was freshly prepared according to Horton (1968) and one unit of cytochrome oxidase activity was defined as that representing a decrease of 0.1 absorbance unit per min at 550 nm. Hydroxypyruvate reductase (HPR) activity was determined as described by Tolbert et al. (1970). The reaction mixture contained 25 ml phosphate buffer (0.02 M, pH 6.2), 0.1 Ml of 4×10^{-3} M NADH, and 0.1 ml of 0.5% Triton X-100. The reaction was initiated with the addition of 0.01 M hydroxypyruvate. All enzyme reactions were assayed at 25° C. Protein was determined according to Lowry et al. (1951).

Enzyme Purification

The CAT-3 isozyme was purified from epicotyls and mesocotyls of 5-day-old etiolated seedlings. For purposes of this investigation, the CAT-1 and CAT-2 isozymes were purified from scutella of 24 h-soaked seed and 10-day-old seedlings, respectively (the developmental time when these isozymes are singly expressed).

Epicotyls (500 mg), mesocotyls (1 kg) or scutella (300 mg) were isolated and homogenized in a Waring blender with 500 ml of 50 mM Tris-HCl buffer, pH 7.4. The homogenate was squeezed through four layers of cheesecloth and centrifuged at $17{,}000 \times g$ for 20 min. The supernatant was fractionated by adding solid (crystal) ammonium sulfate. The catalase was precipitated between 20–40% saturation of ammonium sulfate. The pellet was then resuspended in 15 ml buffer and passed through a Sephadex G-150 column (2.6 cm × 70 cm). Fractions containing catalase activity were pooled and loaded onto a DEAE-Sephadex column (1.5 cm × 30 cm). Catalase was then eluted with a linear 0 to 0.3 M KCl gradient. Fractions with catalase activity were collected, concentrated, and stored at −20° C in buffer containing 30% ethylene glycol. The purification achieved by the above procedures was approximately 80-fold.

The specific activities of the different catalase isozymes purified by these procedures were as follows: CAT-1 (from 1-day-old scutella), 561 units/mg protein; CAT-2 (from 10-day-old scutella), 2,343 units/mg protein; CAT-3 (from epicotyl), 369 units/mg protein; CAT-3 (from mesocotyl), 327 units/mg protein.

Physicochemical Properties

Isoelectric point, thermostability, Km and Aminotriazole inhibition of CAT-3 were determined as previously described for CAT-1 and CAT-2 (Scandalios, et al. 1972). 3-Amino-1,2,4-triazole (AT) inhibits catalase activity by binding irreversibly to the apoenzyme (Margoliash et al., 1960).

Turnover of CAT-3

The rate of CAT-3 turnover was determined using the density labeling techniques previously described (Quail and Scandalios, 1971).

Results

Tissue-Specificity of CAT-3

The CAT-3 isozyme is predominantly, and in some lines exclusively, expressed in the pericarp of 25-day old kernels (Fig. 1) and in the shoot of young seed-

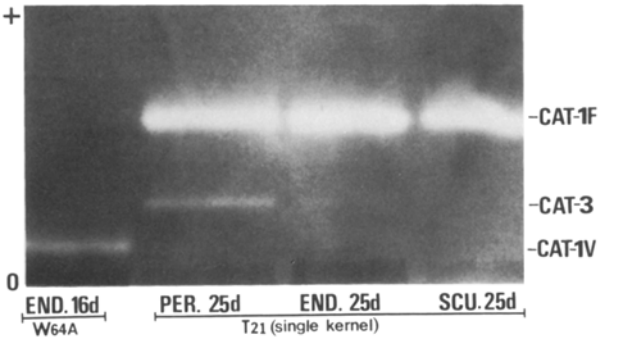

Fig. 1. Zymogram showing the expression of CAT-3 catalase in the pericarp of 25-day old kernels. The pericarp (PER), endosperm (END) and scutellum (SCU) were dissected from a single kernel of the inbred strain T21 (homozygous for the F-allele of *Cat1*). Note that CAT-3 is expressed only in the pericarp, and that it does not interact with CAT-1 to form hybrid molecules. Milky endosperm (END 16d) of W64A (homozygous for the V-allele of *Cat1*) is used as a marker, on the left. Migration is anodal

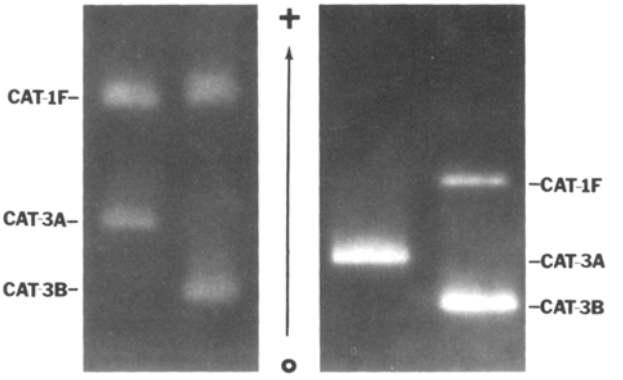

Fig. 3. Zymograms showing that in some inbred lines CAT-1 is co-expressed with CAT-3 in the primary leaf, while in some only CAT-3 is expressed. Note that when both are expressed their subunits do not interact to generate hybrid isozymes. Migration is anodal

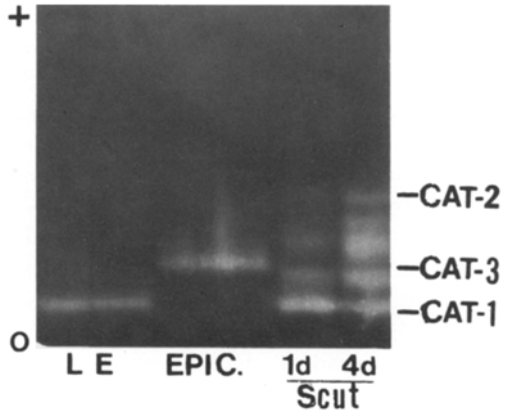

Fig. 2. Zymogram showing the expression of CAT-3 in the epicotyl (EPIC) and its relation to CAT-1 and CAT-2 in the inbred W64A (homozygous for the V-allele at *Cat1* and for the Z-allele at *Cat2*). LE = milky endosperm, 16-days post-pollination, at which time only the *Cat1* gene is expressed; EPIC = epicotyl from 4–7 days after germination, at which time and place only CAT-3 is expressed; SCUT = scutellum, one day (1d) after germination at which time CAT-1 is expressed, but CAT-2 is beginning to be detected by its interaction with CAT-1 to form hybrid catalases, and at four days (4d) after germination when CAT-2 is fully expressed. The intermediate bands are heterotetramers between CAT-1 and CAT-2 (see also Scandalios, 1979). Migration is anodal

lings. Electrophoretically, CAT-3 is easily distinguishable from either the CAT-1 or CAT-2 catalases (Fig. 2). In a few of the large number of inbred lines screened, the CAT-1 and CAT-3 catalases are simultaneously expressed in the same tissue, but their subunits do not interact to form hybrid molecules (Fig. 3). In addition, we have recently found one line, Tx303, in which CAT-3 is co-expressed with CAT-2 in the scutellum and they do not appear to interact to generate hybrid molecules.

Electrophoretic Variants of CAT-3

Two distinct electrophoretic variants of CAT-3 have been found among the numerous different inbred lines we have screened. These variants have been designated as CAT-3A and CAT-3B; the A-variant exhibits a more anodal migration than does the B-variant at pH 8.3 (Fig. 3). Of the inbred strains screened, 86% carried the A-variant and only 14% the B-variant.

Genetic Control of the CAT-3 Variants

The F_1 progeny from crosses made between different inbred strains exhibiting similar CAT-3 phenotypes do not differ from either parental catalase with respect to electrophoretic mobility. However, when crosses are made between strains where one parent carries the CAT-3A variant and the other the CAT-3B variant, the resulting heterozygotes exhibit a fivebanded A/B phenotype (Fig. 4). This suggests that CAT-3, like CAT-1 and CAT-2, is a tetrameric molecule.

Backcrosses yielded parental and hybrid phenotypes in close agreement with the expected 1:1 ratio for simple Mendelian inheritance. The F_2 progeny segregated for the A/A, A/B, and B/B phenotypes in a ratio which statistically fits the expected 1:2:1 ratio.

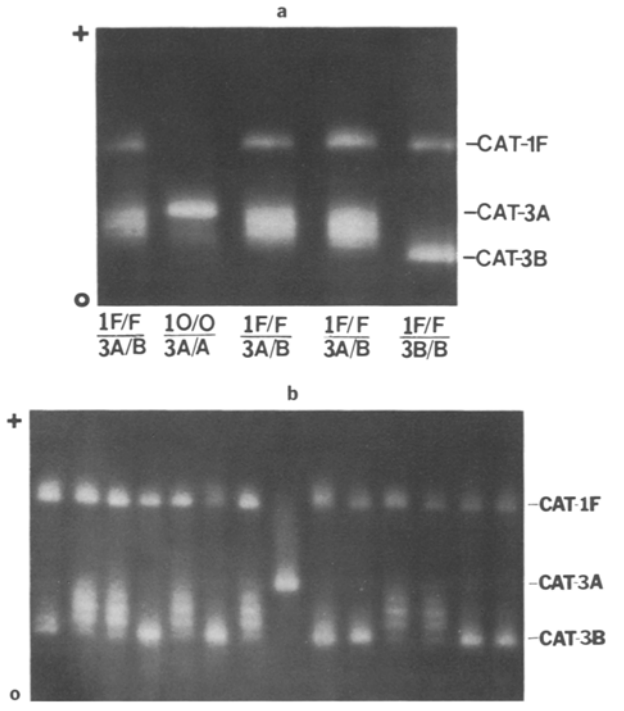

Fig. 4a and b. Zymograms showing phenotypes obtained from genetic crosses between the CAT-3A and CAT-3B variants. Note that CAT-3 segregates independently of CAT-1. **a** Shows results from an F_1 cross (AA × BB). In addition to the parental isozymes, the heterozygotes show three additional isozymes with intermediate electrophoretic mobilities; these are hybrid catalases comprised of both A and B polypeptide subunits. Like CAT-1 and CAT-2, CAT-3 is a tetrameric enzyme. **b** Shows phenotypes from the backcross (BB × AB); CAT-3A was used as a marker. Migration is anodal

Table 1. Results of crosses showing the mode of inheritance of CAT-3 catalase in maize

Parents		CAT-3 phenotypes of offspring						
♀	♂	AA	AB	BA	BB	Total	χ^2	*P*
AA	AA	40				40	–	–
BB	BB				50	50	–	–
AA	BB		40			40	–	–
BB	AA		40			40	–	–
AA	AB	96	77			173	1.873[a]	0.17
BB	AB		58		52	110	0.227[a]	0.63
AB	AA	57	54			111	0.036[a]	0.85
AB	BB		57		53	110	0.082[a]	0.77
AB	AB	74	130		49	253	5.134	0.08
BA	BB			18	16	34	0.029[a]	0.86
BA	AA	15		19		34	0.265[a]	0.61
BB	BA			17	17	34	0.000[a]	
AA	BA	20		14		34	0.735[a]	0.39
BA	BA	15		33	16	64	0.024[a]	0.99

[a] Yates correction employed

The genetic data obtained from all crosses (Table 1) suggest that the two electrophoretic variants, CAT-3A and CAT-3B, are coded by two codominant alleles, *Cat3A* and *Cat3B*, at the *Cat3* gene locus. Homozygous individuals carrying only the *Cat3A* allele produce the CAT-3A isozyme, and homozygotes for the *Cat3B allele* produce only the CAT-3B isozyme. In heterozygotes where both alleles are present, the A and B subunit polypeptides interact randomly to generate the two parental homotetramers and three heterotetramers with intermediate electrophoretic mobilities, as is the case for the CAT-1 and CAT-2 catalases (Scandalios, 1965; 1975).

Intracellular Location of CAT-3

Following equilibrium centrifugation of primary leaf extracts on sucrose gradients, two peaks of organelle associated catalase activity were observed. A minor peak was associated with particles at a density of 1.210 kg/l, which likely represents immature peroxisomes; the second, and predominant, peak of catalase activity coincided with the mitochondrial peak at a density of 1.189 kg/l. The mitochondrial catalase activity is tissue-specific since it was found in primary leaves, but not in scutella isolated from the same population of seedlings (Fig. 5). Recall that CAT-3 is not expressed in scutellar extracts of most common inbreds, but that CAT-1 and CAT-2 are. The purity of mitochondria, peroxisomes (primary leaves), and glyoxysomes (scutella) was determined by the marker enzymes cytochrome oxidase, hydroxypyruvate reductase, and isocitrate lyase (isocitratase), respectively, and by the organelle specific isozymes of GOT, IDH, and MDH (see Fig. 5). In addition, electron micrographs prepared from the mitochondrial fraction (the 1.189 kg/l peak) showed clear mitochondrial bodies without microbody contamination (Fig. 6). Therefore, contamination of the mitochondria with microbodies seems unlikely.

Recovery of Catalase in Submitochondrial Particles

For investigation of the submitochondrial particles, one-tenth of the mitochondrial pellet was taken as a reference in each experiment and the activity of cytochrome oxidase was chosen as a basic parameter of mitochondrial ghosts. Based on the ratio of catalase to cytochrome oxidase, 42% of the catalase activity associated with mitochondria was recovered in submitochondrial particles (Table 2). This result excludes the possibility that mitochondrial associated

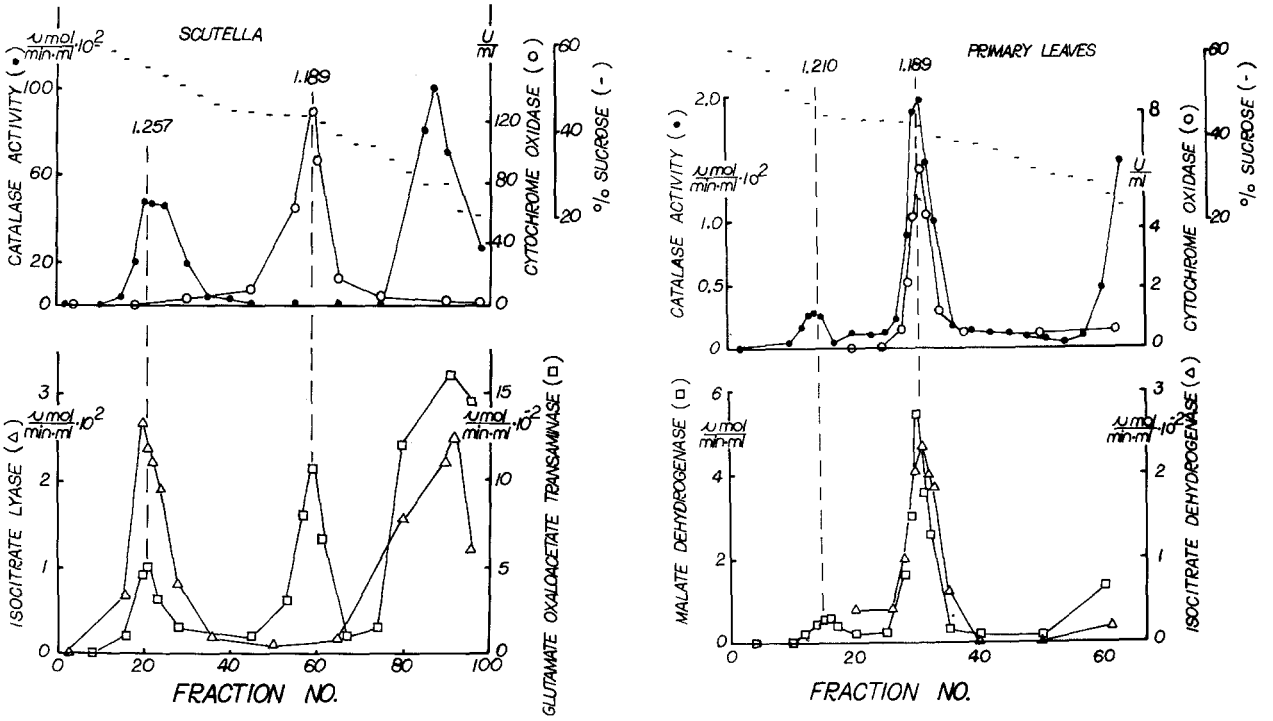

Fig. 5. Separation of organelles from 4-day-old etiolated seedlings by linear sucrose gradients (27 ml, 60-30% sucrose) for scutella, and by stepped gradients (3 ml, 60%; 3 ml, 50%; 9 ml, 43%; 6 ml, 30% sucrose) for primary leaves. In scutella, only one peak of catalase activity ($\rho = 1.26$ kg/l) is present and corresponds to the glyoxysomal peak using isocitrate lyase and the glyoxysomal malate dehydrogenase isozyme as marker enzymes. In primary leaves, there is a predominant catalase activity peak ($\rho = 1.19$ kg/l) which corresponds to the mitochondrial peak using cytochrome oxidase and the mitochondrial isozymes of malate and isocitrate dehydrogenases as marker enzymes. A second minor peak of catalase activity is associated with particles at a density of 1.21 kg/l which probably represents immature peroxisomes since only the peroxisomal MDH isozyme is associated with this fraction

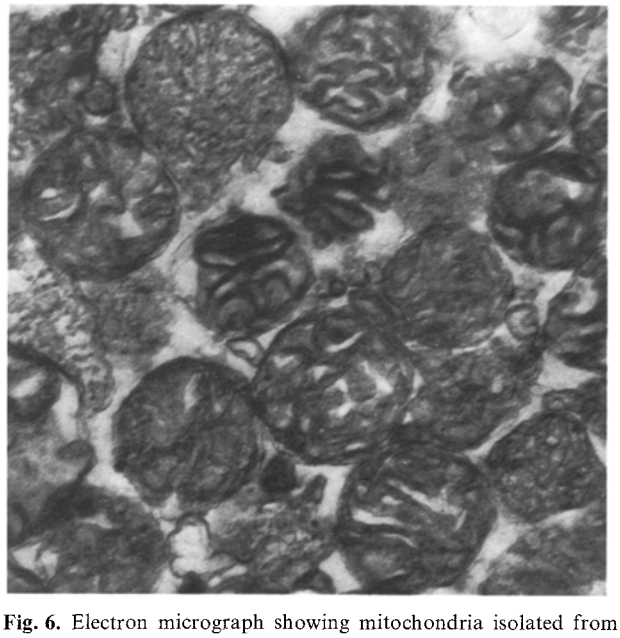

Fig. 6. Electron micrograph showing mitochondria isolated from maize primary leaves (×12,000). Note absence of any microbody particles. Photo is of mitochondria collected from the peak of cytochrome oxidase and coincident catalase fractions in Fig. 5 (primary leaves)

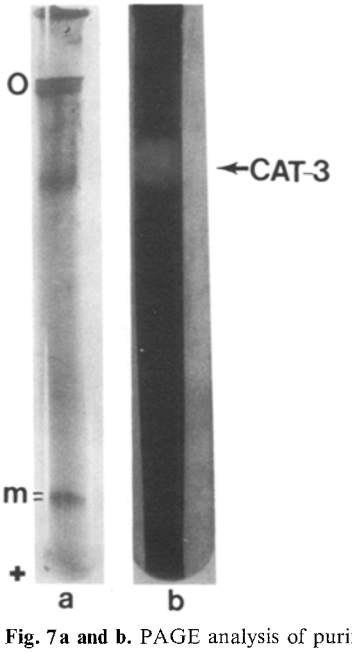

Fig. 7a and b. PAGE analysis of purified CAT-3 catalase. **a** 50 µg of purified CAT-3 stained for protein (Coomasie blue); **b** 60 µg of purified CAT-3 stained for enzyme activity. Note absence of contamination. Migration is anodal; O=origin of sample; m=marker

Table 2. Catalase activity in submitochondrial fractions

Experiments (crude mitochondria)[a]	Fractions	Catalase (U/mg Protein)	Cytochrome oxidase (U/mg Protein)	Catalase/ cytochrome oxidase	% Recovery (relative to cytc. oxidase)
1.	Mitochondria	267.0	26.8	9.96	100.0
	Submitochondrial[c]	90.0	27.3	3.32	33.4
2.	Mitochondria	346.0	13.3	26.02	100.0
	Submitochondrial[c]	276.0	23.5	11.74	44.0
(Purified Mitochondria)[b]					
1.	Mitochondria	25.6	40.0	0.64	100.0
	Submitochondrial[c]	9.2	32.8	0.28	43.8
2.	Mitochondria	105.0	260.0	0.40	100.0
	Submitochondrial	7.7	51.4	0.15	37.5
3.	Mitochondria	88.0	35.7	2.46	100.0
	Submitochondrial	5.7	6.1	0.93	38.0
4.	Mitochondria	93.1	108.1	0.86	100.0
	Submitochondrial	14.9	35.0	0.43	49.0

[a] Mitochondrial fractions obtained by differential centrifugation (9,500 × g) and washed once with buffer
[b] The mitochondrial fractions obtained by differential centrifugation were purified by subsequent sucrose gradient centrifugation
[c] Submitochondrial fractions obtained by osmotic shock of mitochondria

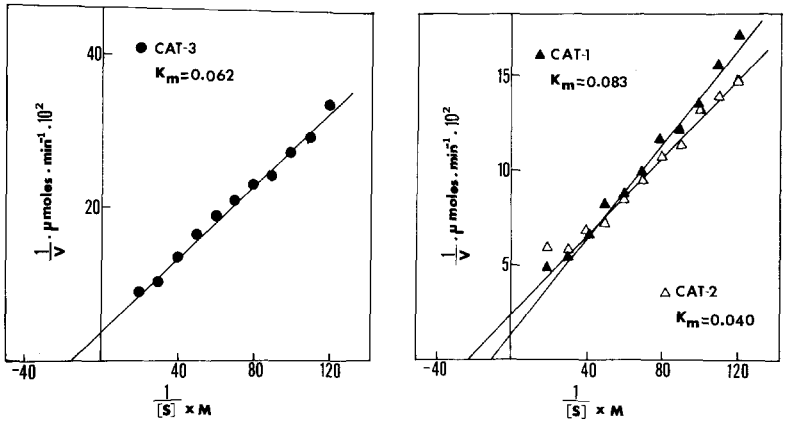

Fig. 8. Double reciprocal plots of 1/V versus 1/S for the reaction catalyzed by catalase at pH 7.0 and 25° C. Purified CAT-3, CAT-2 and CAT-1 catalases were used

catalase in epicotyls is due to microbody contamination and indicates that catalase in the mitochondrion could exist either partly bound to the membrane and partly soluble in the matrix, or in the interspace of the double membrane. In either case, some catalase activity would be lost in the process of forming submitochondrial particles.

Some Biochemical Properties of CAT-3 Catalase

a) Homogeneity of Cat-3 Catalase. Following the purification procedure discussed (see Materials and Methods), the enzyme was subjected to disc gel electrophoresis to determine its purity. Gels stained with Coomasie Blue showed a single protein band; gels stained for catalase activity also showed a single catalase enzyme band which was coincident with the protein band in electrophoretic mobility (Fig. 7).

b) Isoelectric Point (pI). The pI of CAT-3 was determined by isoelectric focusing to be 5.2; this is lower than the pI for either CAT-1 or CAT-2 (Scandalios et al., 1972).

c) Kinetic Constants (K_m). The Michaelis constants were determined for H_2O_2 for CAT-1, CAT-2, and CAT-3. The CAT-2 catalase exhibited the lowest K_m and CAT-1 the highest K_m, while the K_m for CAT-3 was intermediate (Fig. 8). In all cases the K_ms are high to be considered biologically significant, but they are typical for K_m values reported for catalases from a variety of sources.

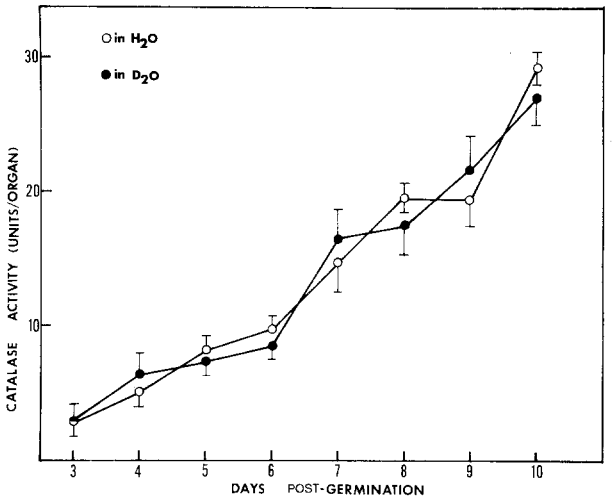

Fig. 9. Time course of catalase activity in epicotyls of etiolated maize seedlings grown on H_2O (—○—) or D_2O (—●—) soaked germination paper. Each point represents the mean ± S.E. of three independent experiments

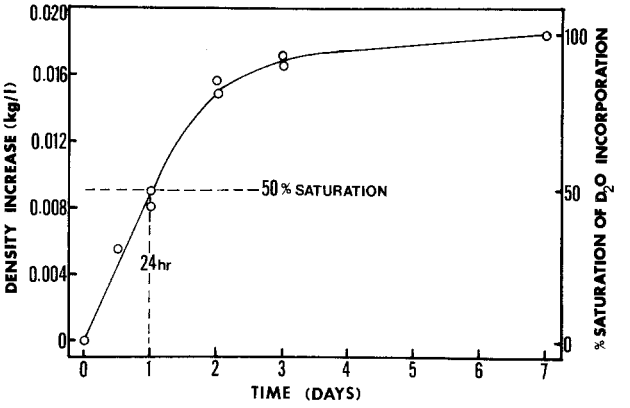

Fig. 11. Kinetics of density of catalase in the epicotyl of etiolated maize seedlings. Seedlings were germinated in H_2O for 48 h then transferred to D_2O (designated as time 0). It is assumed that the maximal density increase (designated as 100% saturation) is reached after the seedlings were incubated in D_2O for 7-days. The dashed lines indicate half saturation of labeling which represents the turnover rate of the enzyme in the epicotyl

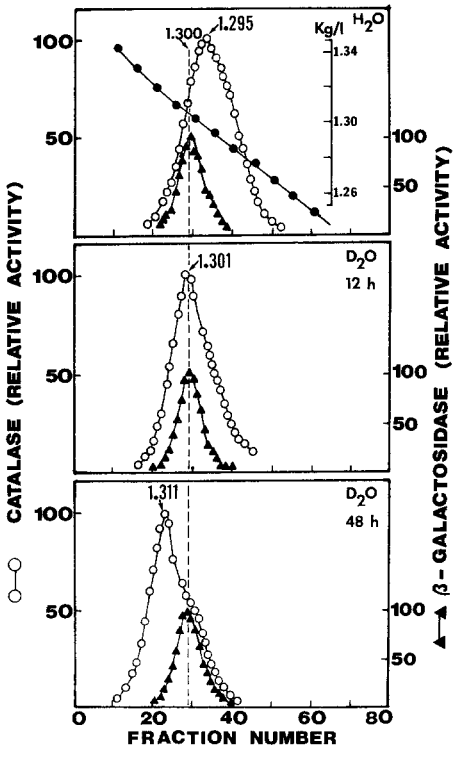

Fig. 10. Equilibrium distribution in CsCl gradients of CAT-3 catalase from maize epicotyl extracts. Seeds were germinated in H_2O and not transferred to D_2O (Control; top); 12 hr in D_2O (middle); 48 h in D_2O (bottom). The activities of the β-galactosidase markers (▲—▲) from each experiment have been lined-up. Relative activity means that all points on these curves are expressed as a percentage of the highest point on each of the individual curves. Density of CsCl gradient (●—●). Note shift in the density peak for catalase indicating incorporation of heavy isotopes

d) Thermostability. The heat stability of CAT-3 catalase purified from isolated epicotyls was measured as an index of its lability in comparison to the CAT-1 ($t_{1/2}=10$ min) and CAT-2 ($t_{1/2}=140$ min) catalases. Like CAT-1 and CAT-2 (Scandalios et al., 1972), the denaturation of CAT-3 with heat treatment follows exponential decay kinetics, and its half-life ($t_{1/2}$) can be recoreded. At 55° C, the CAT-3 catalase has a half-life of about 16 min.

e) Effect of Aminotriazole (AT). Incubation of CAT-3 catalase in 0.1 M 3-amino-1,2,4-triazole for 60 min resulted in approximately 75% inhibition. Thus, like all other catalases, CAT-3 is sensitive to this drug and appears to be more effectively inactivated than the CAT-1 and CAT-2 catalases of maize (Scandalios et al., 1972).

Turnover of the CAT-3 Catalase

Catalase activity increases in the epicotyl and mesocotyl with time during early sporophytic development. The increase in the mesocotyl is slightly more rapid than in the epicotyl but the general time-course profile is the same. The pattern of catalase activity remains the same whether the seedlings are grown in the presence of H_2O or D_2O (^2H_2O, 70 atom %) (Fig. 9). By employing the density labeling technique (Quail and Scandalios, 1971) we have demonstrated that the apparent increase in catalase activity in the epicotyl

is due to the de novo synthesis of the enzyme molecules. The mean density of catalase increases significantly during the course of D_2O incubation (Fig. 10) suggesting synthesis. Kinetic studies indicate that the levels of saturation (physiological saturation) are reached after the seedlings were incubated in 70% D_2O for 7-days (Fig. 11). The time required to reach 50% saturation (representing the rate of turnover) was estimated to be about 24 hr for CAT-3 in the epicotyl. Similar results were obtained for CAT-3 catalase from the mesocotyl.

Discussion

The catalase gene-enzyme system of maize presents an excellent opportunity to examine some of the mechanisms involved in gene regulation and expression during eukaryote development and differentiation (Scandalios, 1979).

In this communication, we have presented evidence for the existence of a catalase isozyme (CAT-3), encoded in the nuclear gene *Cat3*, which is under temporal and spatial regulation. Furthermore, unlike the CAT-1 and CAT-2 catalases of maize which are associated with glyoxysomes in the scutellum (Longo and Longo, 1970; Scandalios, 1974), the CAT-3 isozyme appears to be associated with the mitochondria isolated from young leaves. Mitochondria isolated from both the primary leaf and coleoptile sheath have been found to have significant catalase activity, but mitochondria isolated from scutella from the same seedlings do not show any mitochondrial-associated catalase. The mitochondrial catalase activity of leaves is due to CAT-3 which is not normally expressed in scutella. Preliminary data suggest that the minimal catalase activity associated with pro-microbodies in the young leaves may not be CAT-3, but CAT-1, which in some lines is co-expressed with CAT-3.

That the mitochondrial catalase activity is not merely due to contamination by microbodies is supported by the following: (a) No microbodies can be observed in electron micrographs prepared from mitochondrial fractions (Fig. 6); (b) catalase activity associated with both immature microbodies and mitochondria was observed on the same gradient; (c) microbody-associated catalase has not been found to be a membrane-bound protein. Consequently, catalase from broken membranes accumulates at the top of sucrose gradients (Feierabend and Beevers, 1972); (d) significant catalase activity is recovered in the submitochondrial particles (Table 2) following fractionation of pure mitochondria. This would not be the case if the mitochondrial-associated catalase was due to contamination by microbodies.

Cyanide insensitive respiration has been observed in the mitochondria of many plant tissues (Henry and Nye, 1975). More recently, the production of H_2O_2 and the presence of catalase in the mitochondria of mung bean hypocotyl has been demonstrated (Rich et al., 1976). It has been suggested that catalase prevents the accumulation of H_2O_2 produced by partial reduction of oxygen by the alternate oxidase pathway in plant mitochondria. The existence of cyanide insensitive respiration in etiolated maize epicotyls has been demonstrated (Tong and Scandalios, unpublished data) and the association of catalase with mitochondria has been observed not only in the primary leaves and coleoptiles, but also in the mesocotyls of maize (Tong and Scandalios, 1977). These results suggest that the mitochondrial-associated catalase may be coupled with the alternate oxidase pathway.

Approximately 42% of the total catalase activity associated with the mitochondria was recovered in submitochondrial particles. This suggests that catalase may be located either heterogeneously throughout the mitochondria, or homogeneously in the interspace of the double membranes. Since it has been proposed that the alternate oxidase is located on the outer surface of the mitochondrial inner membrane (Schonbaum et al., 1971), and assuming that catalase is coupled with the alternate oxidase pathway, it seems that the latter possibility would be more likely.

Both catalases are known to be homotetramers, and preliminary evidence, using a previously described technique (Scandalios, 1965), suggests that CAT-1 and CAT-3 can interact, in vitro, to generate heterotetrameric molecules. The fact that these two catalases do not interact *in vivo* may be due to either, (a) that they are compartmentalized in different subcellular organelles, or (b) that CAT-1 and CAT-3 may be localized in different cell lines within the tissues examined.

The biochemical properties of CAT-3 demonstrate that this form of catalase, though similar in some respects, differs significantly from CAT-1 and CAT-2 of maize (Scandalios et al., 1972). The apparent differences in the properties of the different maize catalases may be physiologically significant, and may reflect their *in situ* roles within the cell.

The mechanism of incorporation of most soluble proteins of nuclear gene origin into mitochondria is still unkown. The results presented in this paper raise some important questions. How does catalase become associated with the mitochondria of maize epicotyls, and why is it not associated with the mitochondria of maize scutella? Is this organelle specificity related to the fact that the catalase isozymes exhibit tissue and temporal specificity? What is the molecular (and

genetic) basis of this tissue specifictiy and can it tell us something about the compartmentation process? Further approaches to these questions are currently under investigation.

References

Breidenbach, R.W., Beevers, H.: Association of the glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. Biochem. Biophys. Res. Commun. **27**, 462–469 (1967)

Chance, B., Maehly, A.C.: In: Methods in enzymology, Vol. II (S.P. Colowick and N.O. Kaplan, eds.) pp. 764–775. New York: Academic Press Inc. 1955

Dixon, G.H., Kornberg, H.L.: Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**, 3p (1959)

Feierabend, J., Beevers, H.: Developmental studies on microbodies in wheat leaves. II. Ontogeny of particulate enzyme associations. Plant Physiol. **49**, 33–39 (1972)

Felder, M.R., Scandalios, J.G.: Purification and partial characterization of two genetically defined alcohol dehydrogenase isozymes in maize. Biochim. Biophys. Acta **318**, 149–159 (1973)

Henry, M.F., Nyns, E.J.: Cyanide-insensitive respiration: An alternative mitochondrial pathway. Subcell. Biochem. **4**, 1–65 (1975)

Horton, A.A.: A simple procedure for the preparation of reduced cytochrome C. Anal. Biochem. **23**, 334–335 (1968)

Longo, C.P., Longo, G.P.: The development of glyoxysomes in peanut cotyledons and maize scutella. Plant Physiol. **45**, 249–254 (1970)

Lowry, O.H., Rosebrough, W.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)

Luft, J.H.: Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9**, 409–414 (1961)

Margoliash, E., Novogrodsky, A.: Irreversible reaction of 3-amino-1,2,4-triazole and related inhibitors with protein of catalase. Biochem. J. **74**, 339–348 (1960)

Quail, P.H., Scandalios, J.G.: Turnover of genetically definded catalase isozymes in maize. Proc. Natl. Acad. Sci. USA **68**, 1402–1406 (1971)

Rich, P.R., Boveris, A., Bonner, W.D., Moore, A.L.: Hydrogen peroxide generation by the alternate oxidase of higher plants. Biochem. Biophys. Res. Commun. **71**, 695–703 (1976)

Scandalios, J.G.: Subunit dissociation and recombination of catalase isozymes. Proc. Natl. Acad. Sci. USA **53**, 1035–1040 (1965)

Scandalios, J.G.: Genetic control of multiple colecular forms of catalase in maize. Ann. N.Y. Acad. Sci. **151**, 274–293 (1968)

Scandalios, J.G.: Genetic control of multiple molecular forms of enzymes in plants. Biochem. Genet. **3**, 37–79 (1969)

Scandalios, J.G., Liu, E.H., Campeau, M.A.: The effects of intragenic and intergenic complementation on catalase structure and function in maize: a molecular approach to heterosis. Arch. Biochem. Biophys. **153**, 695–705 (1972)

Scandalios, J.G.: Subcellular localization of catalase variants coded by two genetic loci during maize development. J. Heredity **65**, 28–32 (1974)

Scandalios, J.G.: Differential gene expression and biochemical properties of catalase isozymes in maize. In: Isozymes, III; Developmental biology (C.L. Markert, Ed.) pp. 213–238. New York: Academic Press Inc. 1975

Scandalios, J.G.: Physiological genetics. New York: Academic Press Inc. 1979

Schonbaum, G.R., Bonner, W.D., Storey, B.T., Bahr, J.T.: Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Plant Physiol. **47**, 124–128 (1971)

Smith, L.: Spectrophotometric assay of cytochrome C oxidase. In: Methods of biochemical analysis, Vol. 2 (D. Glick, ed.) pp. 427–434. New York: Interscience Publishing Inc. 1955

Tolbert, N.E., Yamazaki, R.K., Oeser, A.: Localization and properties of hydroxypyruvate and glyoxylate reductases in spinach leaf particles. J. Biol. Chem. **19**, 5129–5136 (1970)

Tong, W.F., Scandalios, J.G.: Catalase isozymes in etiolated epicotyls of maize seedlings: A mitochondrial-specific form of catalase. Isozyme Bull. **10**, 26–27 (1977)

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