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Communication

Enhanced-Peroxidatic Activity in Specific Catalase Isozymes of Tobacco, Barley, and Maize

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ABSTRACT

Separation of catalase isozymes from leaf extracts of three diverse plant species (*Nicotiana sylvestris, Zea mays, Hordeum vulgare* L.) revealed a distinct isozyme with enhanced peroxidatic activity (30-, 70-, 28-fold over typical catalase, respectively) which constituted 10 to 20% of the total catalase activity. In maize this isozyme is the product of the *Cat3* gene, which is expressed only in mesophyll cells (AS Tsaftaris, AM Bosabalidis, JG Scandalios [1983] Proc Natl Acad Sci USA 80: 4455–4459). A mutation in barley reducing levels of peroxisomal catalase (AC Kendall et al. [1983] Planta 159: 505–511) does not reduce the amount of the isozyme with enhanced peroxidatic activity. Similarly, this isozyme is unaffected in dark-grown barley in spite of a 75% decrease in total catalase activity. These results suggest that this catalase isozyme is under separate genetic control in barley. This may also be the case in tobacco where the catalase isozyme with enhanced peroxidatic activity is an immunologically distinct protein (EA Havir, NA McHale [1989] Plant Physiol 89: 952–957).

Catalase (EC 1.11.1.6) can function in the catalatic or peroxidatic modes, catalyzing either the direct decomposition of H_2O_2 to O_2 and H_2O, or the oxidization by H_2O_2 of substrates such as methanol, ethanol, formaldehyde, formate, nitrite, or elemental mercury (6, 11, 17). Although both activities of the enzyme from mammalian sources have been extensively studied (2, 16), the mechanism of action of plant catalases has not been reported.

Examples of developmental regulation of catalase in plant tissue (e.g. 3–5, 8, 9, 15) are known but it has been generally assumed that all forms of the enzyme exhibit the same catalytic properties. However, recently we demonstrated that in tobacco and spinach leaves there are two forms of catalase, distinguishable by their ratios of peroxidatic/catalatic activity (4, 5). In tobacco the peroxidatic activity was enhanced 30- to 50-fold in the form of catalase, designated by its order of elution from a chromatofocusing column, as CAT-3. In spinach, the ratio varied 10-fold for the two types of catalase.

The existence of two types of catalase in tobacco (T-CAT,[1] represented by CAT-1, and EP-CAT, represented by CAT-3) raised the possibility that they were controlled by separate genes. Several lines of evidence indicated that CAT-1 and CAT-3 were controlled separately (4, 5). These included (a) the different time frames during development for expression of activity, (b) the different responses to CO_2-enrichment, and (c) the lack of cross-reactivity of CAT-3 with the antibody raised against CAT-1 (4, 5). Earlier work in maize had shown that there are three genes (*Cat1, Cat2, Cat3*) coding for three catalases (CAT-1, CAT-2, and CAT-3) which are expressed during different stages of development (1, 13–15, 18, 19). In contrast, it has been assumed that there is only one gene for catalase in barley (7).

In this paper we present evidence that the CAT-2 isozyme of maize, located in the peroxisomes of bundle sheath cells (19), is the typical form of catalase displaying low peroxidatic activity. In contrast, the CAT-3 isozyme, located in the mesophyll cells (19), has enhanced peroxidatic activity. We also demonstrate that a high peroxidatic isozyme of catalase is present in barley and that it is not affected by a mutation that virtually eliminates activity of the low peroxidatic isozyme located in the peroxisomes.

MATERIALS AND METHODS

Enzyme Assays and Definition of Units

One unit (U) of catalase catalatic activity, measured as described previously (4), is defined as the amount of enzyme catalyzing the decomposition of 1 μmol H_2O_2 min^{-1}. The peroxidatic activity, measured as the conversion of ethanol to acetaldehyde (5), is defined as the amount of enzyme catalyzing the formation of 1 μmol acetaldehyde min^{-1}. The ratio, $R_{p/c}$, of the two activities is defined as (mU peroxidatic/U catalatic)×10.

Plant Material and Growth of Seedlings

Leaves of wild-type and CAT(−) barley (*Hordeum vulgare* L.), grown in potting mix in Plexiglas boxes in an atmosphere of 1% CO_2/21% O_2 under continuous illumination (80 μE m^{-2} s^{-1}), were harvested at 4 to 8 d postgermination. Leaves of wild-type and the null *Cat2* mutant of maize (*Zea mays*), planted and grown in potting mix in the greenhouse, were harvested at 3 to 4 weeks postgermination. Tobacco seedlings (*Nicotiana sylvestris*) were grown either in air or in 1% CO_2/21% O_2 under the same conditions as the barley and harvested at 3 to 4 weeks postgermination.

Leaves (1.5–2.0 g) of barley, maize, or tobacco were ground in a glass homogenizer with 10 mL of cold 0.05 M K phosphate

[1] Abbreviations: T-CAT, typical or low peroxidatic activity catalase; EP-CAT, enhanced or high peroxidatic activity catalase.

(pH 7.4), containing 15 mg DTT and centrifuged at 15,000*g* for 10 min. The supernatant was equilibrated with starting buffer and chromatofocused (pH 8-5) as described previously (4). Aliquots of the eluate were assayed for both catalatic and peroxidatic activities.

Materials

Seeds of the null *Cat2* maize mutant and antibodies to CAT-3 were supplied by Dr. John Scandalios, North Carolina State University. Seeds of the CAT(−) mutant of barley were supplied by Dr. Janice Turner and Dr. Alfred Keys, Rothamsted Experimental Station.

RESULTS

Assay for Peroxidatic Activity and Ratio ($R_{p/c}$) of Peroxidatic to Catalatic Activities

The correlation of catalatic and peroxidatic activities for CAT-1 and CAT-3 from *Nicotiana sylvestris* is shown in Figure 1. Many factors influence the rate of the peroxidatic reaction, the most important of which is the rate at which H_2O_2 is supplied (11). However, a five-fold increase in glucose oxidase did not increase the rate of the peroxidatic reaction for either isoform (data not shown). This determination is important because it excludes the possibility that the difference between $R_{p/c}$ of CAT-1 and CAT-3 results from different affinities for H_2O_2, *i.e.* the ratio of CAT-1 would approach that of CAT-3 at increased levels of H_2O_2. For the data shown in Figure 1, $R_{p/c}$ for CAT-3 was 10 and for CAT-1, 0.35. These results are in good agreement with those published earlier of 9.2 ± 1.0 and 0.28 ± 0.06 for *N. sylvestris* CAT-3 and CAT-1, respectively (4).

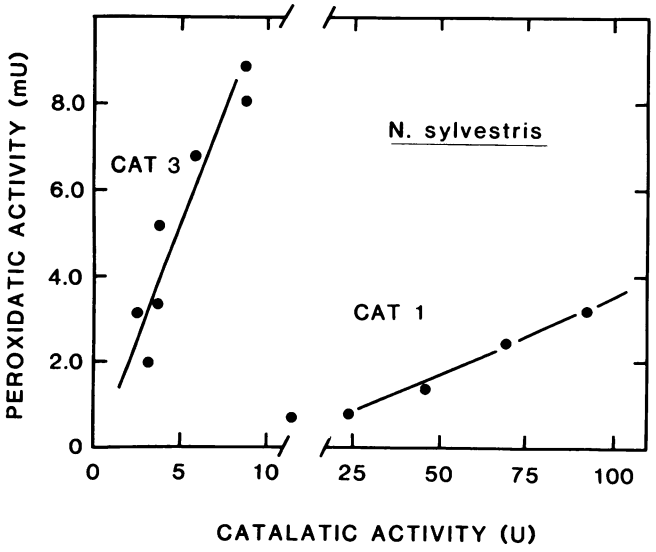

Figure 1. Correlation of catalatic and peroxidatic activities for CAT-1 and CAT-3 from *N. sylvestris*. After chromatofocusing of extracts of *N. sylvestris* fractions comprising CAT-1 and CAT-3 were combined and concentrated to 900 U/mL (CAT-1) or 300 U/mL (CAT-3). Varying amounts of enzyme were assayed for catalatic and peroxidatic activities as described in "Materials and Methods."

Demonstration of an Enhanced-Peroxidatic Catalase in Maize

When extracts of wild-type maize were chromatofocused, two peaks of catalase activity were observed (Fig. 2A), but their identification with the nomenclature of Scandalios (15) was unknown. Most of peroxidatic activity was associated with one form of catalase, the first to elute from the column. Chromatofocused extracts of the null *Cat2* mutant, shown in Figure 2B, had only this first peak of catalase activity, thus corresponding to CAT-3. The identity of the isozymes in wild-type extracts was confirmed by the reaction of protein from the first peak but not the second (Fig. 2A) with anti-CAT-3 antibody (data not shown). The value of $R_{p/c}$ for CAT-3 from mutant and wild-type was 17.6 ± 2.4, and for CAT-2 from wild-type, 0.25 ± 0.05. This establishes that maize leaves possess a catalase isozyme with enhanced peroxidatic activity which is under separate genetic control.

Demonstration of an Enhanced-Peroxidatic Catalase in Barley

Chromatofocused extracts of wild-type barley, shown in Figure 3A, exhibited a shoulder of peroxidatic activity which eluted before the main peaks of catalatic and peroxidatic activity. This is a typical chromatogram for two closely eluting enzymes which differ both in $R_{p/c}$ and in relative amounts. If the catalase with the lower value for $R_{p/c}$ could be reduced, a more definitive separation might be achieved. Since expression of maize CAT-2 (T-CAT) is light dependent but that of CAT-3 (EP-CAT) is not (18), we decided to examine etiolated

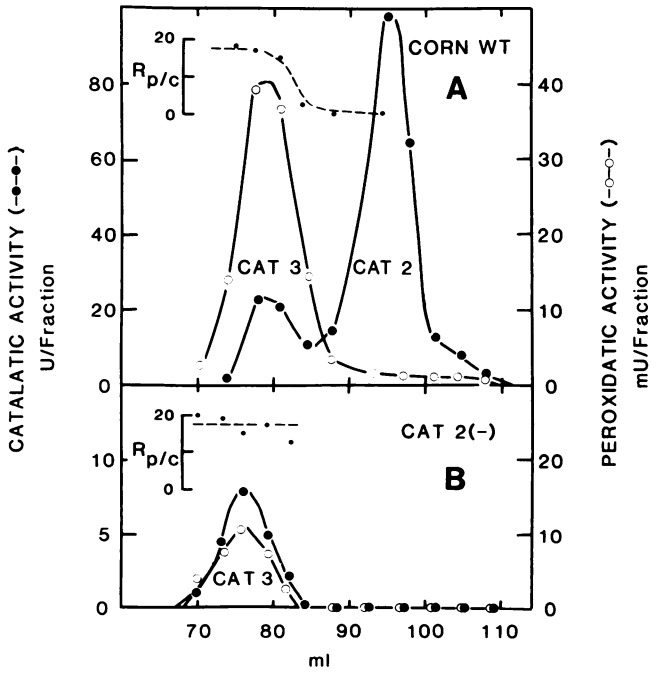

Figure 2. Separation of isozymes of catalase in extracts of wild-type (A) and null *Cat2* mutant (B) maize by chromatofocusing. Total catalatic activity applied to each column was 350 U (A) and 35 U (B). Portions of each fraction were assayed for catalatic and peroxidatic activity. The ratio of the two, $R_{p/c}$, is shown as the dashed line (inset).

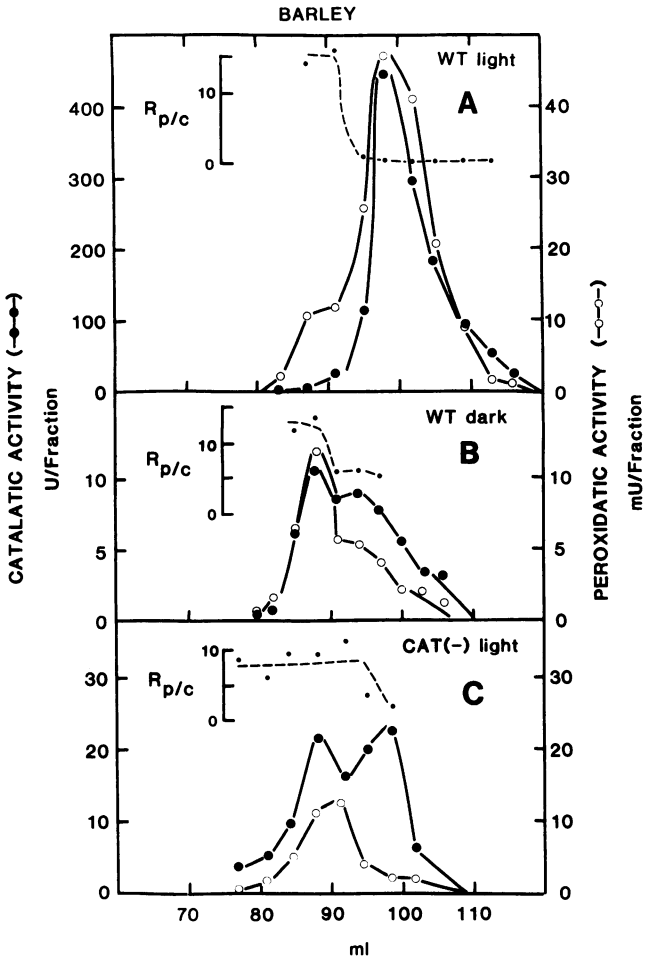

Figure 3. Distribution of catalatic and peroxidatic activities in chromatofocused extracts of wild-type barley grown in the light (A) or dark (B) and CAT(−) barley grown in light (C). Total catalatic activity applied to the column in A, B, and C was 1307, 80, 135 U, representing 3.0, 0.7, and 1.3 g fresh weight, respectively. Portions of each fraction were assayed for catalatic and peroxidatic activity. The ratio of the two, $R_{p/c}$, is shown as the dashed line (inset).

barley for suppression of the T-CAT isozyme. The total catalatic activity in extracts of wild-type barley grown in the dark was reduced 75% and resolved into two closely eluting peaks (Fig. 3B). The values for $R_{p/c}$ for the first fractions are similar to those in Figure 3A. While total catalatic activity was reduced 75%, there was an apparent increase in the peroxidatic activity, *i.e.* 9 mU g^{-1} fresh weight in the light *versus* 36 mU g^{-1} fresh weight in the dark. This increase is probably due in large part to the difficulty of estimating values from chromatograms. We conclude that while the activity of T-CAT is light dependent, that of the EP-CAT is not.

When extracts of CAT(−) barley were chromatofocused (Fig. 3C), two peaks of catalatic activity were obtained, one of which coincided with the main peak of peroxidatic activity. While the leading peak of peroxidatic activity (EP-CAT) was approximately the same in the mutant and the dark-grown wild-type extracts, (*i.e.* 25 *versus* 36 mU g^{-1} fresh weight, respectively), the second peak of catalatic activity (T-CAT) was all but eliminated in the mutant. Determining $R_{p/c}$ for the isozymes is difficult because of their close elution but the ratio $R_{p/c}$ for fractions in the first peak was 11.8 ± 3.2 (EP-CAT) and for fractions in the second peak, 0.42 ± 0.04 (T-CAT). Previously, a ratio of 0.17 ± 0.018 was determined for peak fractions of wild-type barley (4). Together these observations provide evidence for distinct T-CAT and EP-CAT isozymes in barley which may be under separate genetic controls.

A summary of the values for $R_{p/c}$ and identification of EP-CAT and T-CAT isozymes in maize, tobacco, and barley is given in Table I.

DISCUSSION

It has become increasingly clear that catalase activity in plants is not represented by a single type of enzyme. In tobacco, spinach, maize, and barley we have established the existence of a distinct catalase isozyme with enhanced peroxidatic activity (4; this paper). The contribution of EP-CAT to total catalase activity although low is not trivial. For example, in wild-type maize at the stage sampled the contribution of EP-CAT (CAT-3) to total catalase is 20% (data taken from Fig. 2). Determining the distribution of activity between the two isozymes in barley is difficult because of their close elution from the chromatofocusing column but the EP-CAT probably constitutes 5 to 10% of the total activity. The exact amount of EP-CAT in tobacco is also hard to assess because, as we have shown previously, the amount can vary according to age of seedlings (4). However, a reasonable estimate for mature greenhouse-grown leaves, based on data from a number of chromatofocusing columns, is 10% of the total (data not shown).

Certain similarities among the isozymes of catalase with enhanced-peroxidatic activity are emerging. For example, EP-CAT from tobacco and maize is more sensitive to temperature than is T-CAT (1, 4). The expression of T-CAT from both maize and barley is light dependent but that of EP-CAT is not (18; this paper), and maize EP-CAT has been shown to be less sensitive to the inhibitor 3-amino -1,2,4-triazole than T-CAT (1). Several atypical catalases from microorganisms and photosynthetic bacteria also exhibit greater sensitivity to temperature and relative insensitivity to the same inhibitor (10). However, the enzymes from microorganisms can utilize *o*-dianisidine in the peroxidatic reaction (10) whereas EP-CAT from tobacco cannot (our unpublished data).

Table I. *Identification of Isozymes of Catalase with Enhanced-Peroxidatic Activity*

Identification of the isozymes of catalase with enhanced-peroxidatic activity and values for Rp/c (as defined in "Materials and Methods") taken from the text are summarized below.

Species	Ratio: Peroxidatic / Catalatic		Reference
	Enhanced-peroxidatic catalase	Typical catalase	
Maize	17.6 (CAT-3)	0.25 (CAT-2)	(15); This paper
Tobacco	9.2 (CAT-3)	0.28 (CAT-1)	(4)
Barley	11.8	0.42	This paper

In maize, EP-CAT is the product of the *Cat3* gene (14, 15). In other plant species such as tobacco (4, 5) and barley the evidence for separate genetic control is indirect. In barley, reduction of the level of T-CAT catalase in the CAT(−) mutant or in dark-grown wild-type does not affect the EP-CAT form. The CAT(−) mutant displays residual T-CAT activity, presumably resulting from a leaky mutation in this gene. It has not been determined whether the CAT(−) barley produces normal amounts of partially inactive catalase protein but in the null *Cat2* mutant of maize, the CAT-2 protein is absent (19).

It is not known whether the high-peroxidatic form of catalase is located in the peroxisome or elsewhere in the cell. The inability of Parker and Lea (12) to locate residual catalase cytochemically in leaves of the CAT(−) barley mutant might be due to either its low level of activity or its lability. It has been well documented in maize that CAT-2 and CAT-3 are cell specific, *i.e.* CAT-2 (T-CAT) is located in the bundle sheath cells and CAT-3 (EP-CAT) in the mesophyll cells (15, 19). Mesophyll cells contain few peroxisomes and an association of catalase with isolated mitochondria and submitochondrial particles led to the suggestion that catalase was located in the mitochondria (14). Since this isolation was from leaves of the CAT-2(−) mutant, the type of catalase was the high-peroxidatic form (CAT-3).

One of the most intriguing, and as yet unanswered, questions concerns the possible role of EP-CAT in plant metabolism. Certainly the importance of peroxisomal catalase (T-CAT) is underlined by the observation that the CAT(−) mutant of barley cannot survive under photorespiratory conditions (7). Whether an equally important role for EP-CAT can be assigned awaits determination of location and elucidation of fundamental properties.

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