Purification of a Catalase Inhibitor from Maize by Affinity Chromatography[1]

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SUMMARY

A substance has been isolated from maize scutella which inhibits both maize and bovine liver catalase in vitro, but which does not affect maize peroxidases, a group of catalytically related hemoproteins. The substance has been purified by affinity chromatography on immobilized catalase. The inhibitor is both heat labile and non-dialyzable, suggesting it is a protein.

Maize catalase (H_2O_2 :H_2O_2 oxidoreductase, E.C. 1.11.1.6) appears to be regulated by a number of diverse mechanisms during the first several days of seed germination. Although a single structural gene is expressed in the immature kernel, at or about the time of seed maturation a second locus begins to be expressed (3,6). The isozymes encoded by these two loci have distinct physicochemical and kinetic properties (4). The isozymes also have different turnover rates, and these rates appear to be a major factor controlling the expression of the two loci (1,3). During the same developmental period, the levels of catalase in the maize seed also appear to be influenced by increasing compartmentation in glyoxysomes (5), and the preferential secretion of one isozyme from excised scutella in response to the plant hormone gibberellic acid (7). The presence of a catalase inhibitor in maize was first suggested by anomalous recoveries during the purification of catalase from 24 hour imbibed seeds. No such anomalies were observed in four day seeds. In this communication we describe the purification of this inhibitor by affinity chromatography on immobilized catalase and report on its preliminary characterization.

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MATERIALS AND METHODS

Immobilization of the catalase: We have taken advantage of the affinity of the maize inhibitor for bovine liver catalase, which is commercially available in crystalline form, in the purification procedure. For preparation of the gel, 1.5 grams of cyanogen bromide-activated sepharose (Pharmacia Fine Chem.) were washed by suspension in five 50 ml aliquots of 1 mM HCl. Washes were removed by vacuum filtration. After removal of the final wash, the gel was washed in the funnel with 15 ml of 0.1 M bicarbonate buffer of pH 8.5. The gel was then suspended in an enzyme coupling solution, which consisted of approximately 7000 units of bovine liver catalase (2x crystallized, Sigma) in 10 ml of the bicarbonate buffer. The suspension was incubated on a reciprocating shaker at room temperature. After three hours, the coupling solution was removed by vacuum filtration. No catalase activity was found in the filtrate. The gel was then washed with 50 ml of bicarbonate buffer, and with eight alternating 20 ml volumes of acetate buffer (0.1 M, pH 4.0) and borate buffer (0.1 M, pH 8.5). Both buffers contained 1 M NaCl. The gel was resuspended in bicarbonate buffer. The activity of the immobilized catalase was assayed using a modification of the polarographic method previously described (4). A special reaction vessel was constructed to allow the introduction of solid gel through a port in the side of the chamber. For assay, the excess buffer in a slurry of the gel was removed by vacuum filtration. A small portion (approximately 10 mg) of the partially dried gel was weighed and introduced into the reaction vessel on a microspatula. Using this method, assays are reproduceable to within 5%. Repeated partial drying of the catalase-Sepharose gel had no effect on the activity of the enzyme. Using the coupling procedure described above, we routinely obtain specific activities of 5-6 catalase units per mg of gel, which is convenient for analytical purposes. For preparative purposes, the amount of catalase

in the coupling solution is increased to approximately 100,000 units per 10 ml of solution, and specific activities of 40-50 units per mg of gel are generally observed. Following coupling, all gels are stored at 0-4°C in bicarbonate buffer for at least one week to allow for the inactivation of any remaining reactive sites on the gel matrix Chromatography: Sufficient catalase-Sepharose slurry was placed in a chromatographic column (1 cm dia.) to give a packed bed volume of 2.5 ml. Column effluent was monitored through a flow cell at 280 nm. The column was washed with 80 ml of 0.01 M phosphate buffer at pH 7.0. The scutellar extracts were prepared by grinding 8-10 grams of 24 hr. scutella with sand in 30 ml of the phosphate buffer. Seeds used in these experiments were from the highly inbred line 229 (3). The extracts were centrifuged at 10,000 xg for 20 minutes. Lipid material was removed from the surface of the extract and the supernatant was applied to the affinity column. Unbound proteins were eluted from

Table 1. Nonadditivity of Catalase Activities in Mixed Scutellar Extracts

	Catalase Activity (U/ml)
Day 1 Extract	5
Day 4 Extract	281
Expected Activity of a 1:1 mixture*	142
Observed Activity of a 1:1 Mixture	62
% Inhibition	66%

*Expected Activity = (Day 1 Activity + Day 4 Activity)/2

Table 2. Specificity of Inhibition

A Source of Enzyme (Inhibitor substrate)		B Day 1 Maize Extract (containing inhibitor)	Expected Activity[1] of 1:1 Mixture (A+B)	Observed Activity of Mixture	% Inhibition
Day 4 Maize Extract:					
(a) catalase activity[2]	73	144	109	62	43
(b) peroxidase activity[3]	68.6	12.5	40.6	41.0	0
Bovine Liver					
(a) catalase activity	77	144	111	69	38

[1]Expected activity = (A+B)/2; Day 4 maize extract was diluted approximately 1:3 with buffer for standardization of enzyme activities
[2]Catalase activity = micromoles O_2 evolved/min
[3]Peroxidase activity = $\Delta OD_{460}/min \times 10^3$

the column with 0.01 M phosphate buffer at pH 7.0. After the absorbance at 280 nm had returned to baseline, the inhibitor was eluted from the column with 1 M NaCl.

For preparative purposes, larger extract volumes are passed through immobilized catalase of higher specific activity in a batch method using a Buchner funnel. Unbound proteins are washed off the gel (typical gel volume is approximately 10 ml) with 750 ml of 0.01 M phosphate buffer at pH 7.0 prior to elution of the inhibitor with 15 ml of 1 M NaCl. The inhibitor preparation (the NaCl wash) is dialyzed against the phosphate buffer overnight prior to use.

Enzyme Assays: Catalase was assayed, polarographically as previously described (4). Peroxidase activity in the extracts was determined by monitoring the oxidation of o-dianisidine at 460 nm (2).

RESULTS AND DISCUSSION

As shown in Table 1, when the 10,000 xg supernatants from 1 and 4 day scutellar extracts are mixed in equal volumes, the catalase activity of the mixture is less than the sum of the activities added. The unmixed extracts show less than a 5% loss of activity upon standing for several hours at room temperature. The activity of the mixture is also stable after the initial inhibition reaction, which takes less than 5 minutes. This argues strongly against proteolysis as the source of the non-additivity observed in the mixing experiment. Simple dilution effects can also be eliminated since both 1 and 4 day extracts can be diluted by a factor 2 orders of magnitude greater than is employed in the mixing experiments with no loss in enzyme activity.

Although extensive investigations have not yet been completed on the specificity of the inhibitor, the fact that it inhibits bovine liver catalase nearly as effectively as maize catalase, but does not inhibit maize peroxidases, a group of catalytically related hemoproteins (Table 2), suggests that it may be highly specific.

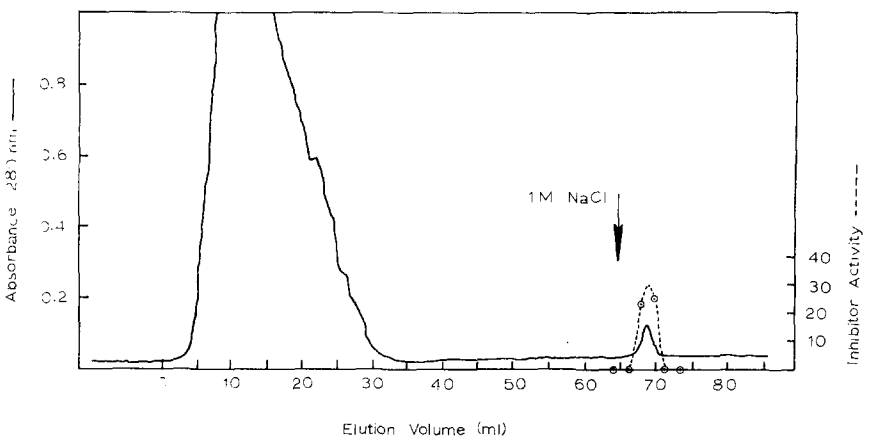

Figure 1. Elution profile of a crude Day 1 scutellar homogenate through the catalase-Sepharose column. The peak of U. V. absorbing material eluted by the NaCl is coincident with inhibitor activity (dashed line). Bed volume of the column is 2.5 ml.

A typical elution profile of a 24 hour extract through the catalase-Sepharose column is shown in Figure 1. Following elution of the bulk of the scutellar proteins through the column, a small peak of U. V. absorbing material can be eluted with 1 M NaCl. As shown by the dashed line, this peak is coincident with the peak of inhibitor activity. Unsubstituted Sepharose showed no ability to bind inhibitor.

If the immobilized catalase is removed from the column at various points in the procedure, washed with buffer, and assayed for catalase activity, it can be seen (Table 3) that the activity of the immobilized catalase is significantly depressed following incubation with the 24 hour extract (due presumably to the binding of inhibitor), and that the activity is restored by washing with NaCl.

Experiments with crude 24 hour homogenates show that the inhibitor is non-dialyzable . We have since confirmed that the purified inhibitor is also non-dialyzable.

The inhibitor is also heat labile (Table 4). These facts, in conjunction with the apparent absorbance of the inhibitor at 280 nm, suggest that it is a protein.

Table 3. Activity of the immobilized catalase during the inhibitor purification procedure.

	Catalase Units/mg gel
Prior to incubation with 24 hr. extract	4.10 ± 0.16*
After incubation with 24 hr. extract	3.68 ± 0.21
After NaCl wash	4.20 ± 0.29

*Standard deviations of a total of seven replicates in two independent experiments utilizing the same batch of immobilized catalase.

Table 4. Heat Lability of the Inhibitor

	Catalase Activity	% Inhibition
Control (Substrate catalase + buffer)	67.2 ± 2.9	-
Heated Inhibitor * + substrate catalase	70.8 ± 1.4	0
Unheated Inhibitor + substrate catalase	37.1 ± 4.9	44.8

* Heated at 50°C for 40 min.

Preliminary gel filtration on G-100 columns indicate that it has a molecular weight of approximately 20,000. The system may be ideal for the study of protein-protein interactions and of enzyme structure-function relationships. Each of the catalase subunits presumably possesses at least two subunit binding sites (the enzyme is a tetramer), as well as an inhibitor binding site which may or may not be distinct from the active center. A number of well defined allelic and non-allelic variant

catalases are available in maize to facilitate such studies.

The probable proteinaceous nature of the inhibitor is also of considerable genetic interest in that recovery of inhibitor variants should enable us to define the gene responsible for inhibitor expression. This, in turn, would allow complete characterization of a post-translational enzyme regulating mechanism at the genetic, biochemical, and developmental levels.

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