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# GLYCOSYLATION OF CATALASE INHIBITOR NECESSARY FOR ACTIVITY[1]

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## SUMMARY

A protein isolated from maize scutella which inhibits catalase *in vitro* has been shown to contain 12% carbohydrate in the form of galactose. This corresponds to four galactose molecules per inhibitor subunit. Removal of the carbohydrate with β-galactosidase or blockage with a galactose-specific lectin abolished activity of the inhibitor.

Maize catalase ($H_2O_2$:$H_2O_2$ oxidoreductase, E.C. 1.11.1.6) appears to be regulated by a number of mechanisms during the first several days of germination. Although a single structural gene is expressed in the immature kernel, a second locus begins to be expressed shortly after imbibition of the mature seed (1). The isozymes have different turnover rates (2, 3) and this appears to be a major factor controlling the expression of the two loci. During the same developmental period, a substance can be detected which inhibits the activity of the catalase isozymes, and follows an inverse activity profile with the enzyme (4). The substance can be isolated by affinity chromatography on immobilized catalase (5). It is a protein with a subunit molecular weight of 5600 daltons which forms a functionally active dimer (6). In this report, we show that the protein contains four galactose residues per subunit which are necessary for activity.

## MATERIALS AND METHODS

Materials: All studies utilized the maize inbred line W64A. Enzymes used in this study were α-galactosidase (*Aspergillus*), β-galactosidase (*E. coli*), α-Mannosidase (jack bean), β-glucosidase (almonds), Neuraminidase (influenza virus) and bovine liver catalase, 2x crystallized. The two lectins used were Concanavalin A and Castor Bean Agglutinin Type II. All enzymes were purchased

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from Sigma with the exception of neuraminidase which was purchased from Calbiochem.

Enzyme assays: Catalase was assayed polarographically as previously described (7). β-galactosidase was assayed as described by Felton et al. (8). Protein was determined by the method of Lowry et al. (9) using bovine serum albumin as the standard.

Inhibitor purification and assay: The inhibitor was purified by affinity chromatography using bovine liver catalase attached to cyanogen bromide activated Sepharose as described previously (5) except that the extract was applied to a 100 x 2.5 cm Biogel P60 column equilibrated with 0.1 M Tris-HCl at ph 7.6 prior to the affinity step. The inhibitor was assayed using partically purified maize catalase or 2x crystallized bovine liver catalase as previously described (4). The inhibitor was electrophoretically homogeneous on SDS polyacrylamide gels (6).

Treatment of Inhibitor with Glycosidases: Aliquots of the purified inhibitor containing 5-10 μg of protein in 0.1 M Tris-HCl, pH 7.6 were incubated for 10 minutes at 25°C with one of the following: 0.60 units of β-galactosidase, 0.1 units of α-galactosidase, 0.36 units of α-mannosidase, 0.07 units of β-glucosidase, 6 units of neuraminidase, or with buffer as a control. After the incubation period, approximately 50 units of catalase were added, and the mixture was incubated for an additional 15 minutes. The mixture was then assayed for catalase activity and compared to the control with untreated inhibitor plus catalase and to the control without inhibitor to determine percentage inhibition. None of the glycosidases tested had any measurable effect on catalase activity.

A dose dependent sensitivity of the inhibitor to β-galactosidase was demonstrated by treating the inhibitor as described above with different amounts (0.60-4.2 units) of β-galactosidase, then assaying for inhibitor activity, or by incubating the inhibitor with 0.60 units of β-galactosidase for varying lengths of time prior to assay.

Sensitivity of the inhibitor to lectins: Concanavalin A (specific for glucosyl and mannosyl residues, ref. 10) or *Ricinus communis* lectin type II-A (specific for galactosyl residues) were dissolved in 0.05 M potassium phosphate, pH 7.0, at a concentration of 0.5 mg per ml. Aliquots containing 10 μg of inhibitor were mixed with 40 μl of the lectin solutions or with buffer as a control. After a 24 hour incubation at 4°C, the mixture was assayed for inhibitor activity. The two lectins had no measurable effect on catalase activity.

Chromatographic analysis of carbohydrate: The identity of the inhibitor glycosides was verified by thin layer chromatography. One ml of purified inhibitor containing approximately 100 μg of protein was dialyzed extensively against 0.05 M potassium phosphate, pH 7.0. The sample was then hydrolyzed with 0.1 N $H_2SO_4$ for 48 hours at 100°C, neutralized and applied to a silica N-HR uv254 thin layer plate. Sugar standards were applied at 0.25 μmoles per sample and the chromatograms were developed for 12 hours with a 9:6:3:1 mixture of n-butanol:acetic acid:ethyl ether:water. The chromatogram was then dried and stained with a 0.5% solution of potassium permanganate in 1 N sodium hydroxide. Aliquots of inhibitor before and after treatment with β-galactosidase were applied to the same chromatographic system.

Determination of percentage carbohydrate: Total carbohydrate in aliquots containing approximately 30 μg of the purified inhibitor was determined by the phenol-sulfuric acid method of Dubois et al. (11) as modified by Ashwell (12) using redistilled reagent grade phenol. The standard curve was established using D-galactose and was linear over the range of 1-10 μg used in this study.

Table 1. Effects of various glycosidases on the catalase inhibitor.

| Glycosidase treatment of inhibitor | Catalase activity* | Catalase + treated inhibitor | % inhibition of catalase |
|---|---|---|---|
| 1. None | 12.4 ± 0.4 | 7.6 ± 0.2 | 38.7 |
| 2. α-galactosidase | 12.2 ± 0.5 | 7.2 ± 0.7 | 40.9 |
| 3. β-galactosidase | 13.2 ± 0.4 | 13.0 ± 1.2 | 0 |
| 4. β-galactosidase† | 15.1 ± 0.5 | 11.9 ± 0.7 | 21.2 |
| 5. β-glucosidase | 12.4 ± 0.5 | 7.0 ± 0.9 | 43.6 |
| 6. α-mannosidase | 12.3 ± 0.5 | 7.6 ± 1.0 | 38.2 |
| 7. Neuraminidase | 12.0 ± 0.5 | 7.8 ± 0.3 | 35.0 |

*Catalase units per ml after 10 minutes incubation with glycosidase listed for inhibitor treatment.

†In this instance, catalase was incubated with untreated inhibitor for 10 minutes, then β-galactosidase was added and the incubation continued for an additional 10 minutes.

## RESULTS AND DISCUSSION

The results of glycosidase treatment on inhibitor activity are presented in Table 1. The only enzyme tested which shows any effect on the inhibitor activity is β-galactosidase, which completely eliminates inhibitor activity. Catalase itself also appears to be affected by this enzyme showing a significant enhancement (approximately 8%) over untreated controls. None of the other glycosidases have any measurable effect on catalase activity. This enhancement can be most reasonably explained by assuming that the catalase preparation contains some molecules which are already complexed with inhibitor. This inhibitor may be inactivated by the β-galactosidase resulting in an increase in the number of active catalase molecules present. The ability of β-galactosidase to partially inactivate inhibitor already bound to catalase can in fact be demonstrated experimentally (Table 1, line 4). In this experiment, β-galactosidase was added after the catalase and inhibitor had been allowed to complex fully, and the treatment results in a partial restoration of catalase activity. Maize catalase itself contains no detectable carbohydrate (data not

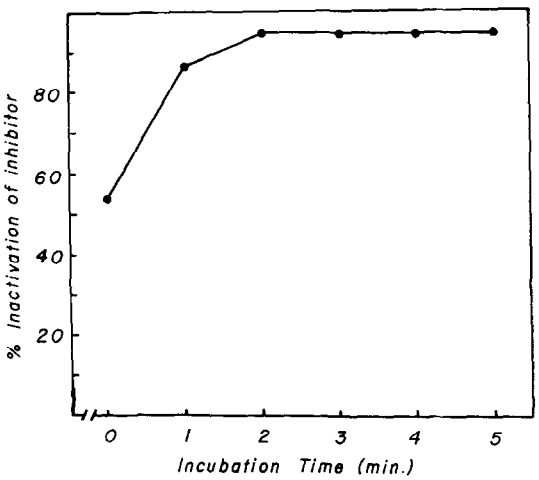


Figure 1. Time course of inhibitor inactivation by β-galactosidase. Aliquots of inhibitor were incubated with 0.6 units of β-galactosidase for the periods of time indicated. The treated inhibitor was then mixed with catalase and incubated for an additional 10 minutes, then assayed for catalase activity. Percent inhibition was determined relative to a control in which catalase was incubated for 10 minutes with buffer.

shown), which further argues that the apparent enhancement of catalase by β-galactosidase may be an indirect effect. We have treated catalase with β-galactosidase, separated the enzymes by gel filtration, and used this pretreated catalase in the inhibitor assay. No anomalous behavior was observed.

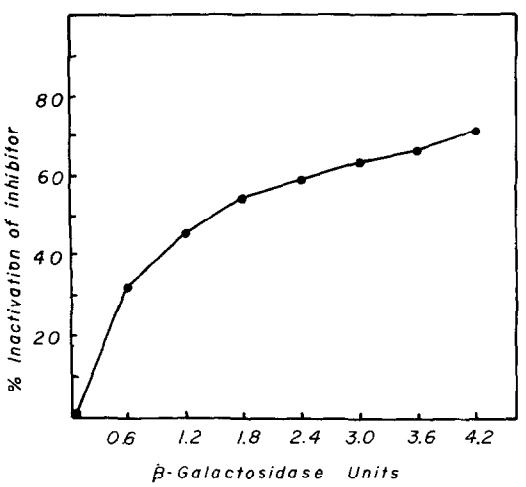


Figure 2. Dose dependent sensitivity of the inhibitor to β-galactosidase. Aliquots of 5 μg of inhibitor were incubated for 10 minutes with the amounts of β-galactosidase indicated, then the inhibitor was assayed against catalase as described in Figure 1.

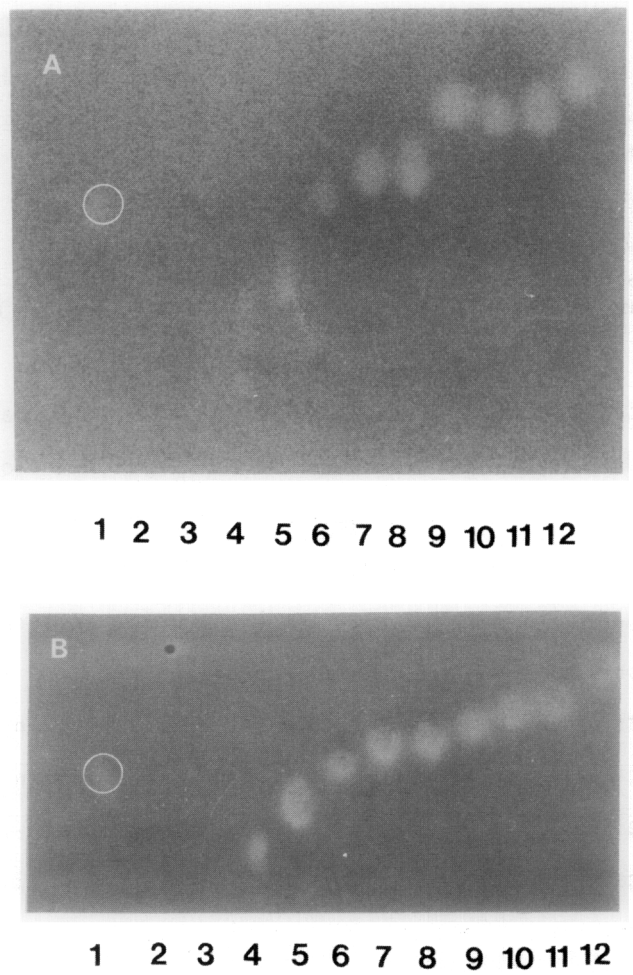


Figure 3. Thin layer chromatographic analysis of inhibitor glycosides released by acid hydrolysis (A) and β-galactosidase (B). (A) Sample 1 represents inhibitor treated with sulfuric acid for 48 hours at 100°C. Other samples in (A) in order are: untreated inhibitor, $H_2SO_4$, galactosamine, sucrose, galactose, glucose, mannose, xylose, fucose, ribose, rhamnose. (B) Sample 1 represents inhibitor treated with β-galactosidase as described in the text. Other samples in order are: untreated inhibitor, β-galactosidase, galactosamine, sucrose, galactose, glucose, mannose, ribose, fucose, xylose, rhamnose.

The inactivation of the inhibitor by β-galactosidase is both time dependent (Figure 1) and dose dependent (Figure 2). Since the inhibitor is incubated with catalase for 10 minutes during the inhibitor assay, zero preincubation time actually represents a 10 minute total incubation with β-galactosidase. This accounts for the loss of inhibitor activity at zero preincubation time in Figure 1.

Table 2. Effects of Lectins on the catalase inhibitor.

| Lectin used to treat inhibitor | Catalase activity* | Catalase + treated inhibitor | % inhibition of catalase |
|---|---|---|---|
| 1. None | 17.2 ± 0.9 | 11.7 ± 0.6 | 31.9 |
| 2. Castor Bean | 17.7 ± 1.0 | 15.9 ± 0.9 | 10.2 |
| 3. Concanavalin A | 17.4 ± 0.9 | 10.8 ± 1.0 | 37.9 |

*Catalase units per ml after a 10 minute incubation with the lectin listed for inhibitor treatment.

The fact that treatment of the inhibitor does in fact release galactose is demonstrated in Figure 3A. A spot on the thin layer chromatogram corresponding to galactose is observed in the sample of inhibitor treated with β-galactosidase but not in the untreated control. The total acid hydrolyzate analysis in Figure 3B shows that galactose is the only carbohydrate detectable in the inhibitor preparation.

The effects of two lectins, Concanavalin A and Castor Bean lectin Type II on inhibitor activity are shown in Table 2. Castor bean lectin which is specific for galactosyl residues inactivates nearly 80% of the inhibitor activity. Concanavalin A, with an affinity for glucose and related residues has no significant effect on inhibitor activity.

Total carbohydrate analysis of the inhibitor using the phenol-sulfuric acid method (11, 12) showed an average of 4.0 μg of galactose for every 32.5 μg of inhibitor. This corresponds to 12.3% carbohydrate and, based on an inhibitor subunit molecular weight of 5600 daltons (6), indicates that there are 3.8 galactose residues per subunit.

Proteinaceous enzyme inhibitors have been reported in a number of plant systems (13 - 17). In most cases, little is known about the physiological functioning of these molecules or about their precise role in controlling enzyme expression. The observation reported here, that a specific carbohydrate is necessary for biological activity of the maize catalase inhibitor, may help answer these basic questions and suggests an additional experimental approach to the problem of enzyme regulation in general.

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