Plant Physiol. (1993) 103: 477–483

Purification and Characterization of Catalase from Loblolly Pine (*Pinus taeda* L.) Megagametophytes[1]

Robert T. Mullen and David J. Gifford*

Department of Botany, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Catalase (EC 1.11.1.6) was purified to near homogeneity from isolated megagametophytes of germinated loblolly pine (*Pinus taeda* L.) seeds, and monospecific antibodies were elicited in rabbits. Following a procedure that involved acetone extraction, $(NH_4)_2SO_4$ fractionation, and four chromatographic steps (i.e. DE-52 cellulose, Superdex-200, hydroxylapatite, and phenyl-Sepharose CL-4B), catalase was purified about 140-fold to a final specific activity of 2215 mmol min^{-1} mg^{-1} of protein. Cotton isocitrate lyase antibodies were used, and protein immunoblots revealed that the resolution on hydroxylapatite and phenyl-Sepharose allowed for the complete separation of catalase from contaminating isocitrate lyase. The molecular masses of the native enzyme and its subunit are 235 and 59 kD, respectively, indicating that the pine holoenzyme is a homotetramer. Loblolly pine catalase exists as multiple isoforms. When megagametophytes taken 7 d after imbibition at 30°C were extracted, subjected to nondenaturing isoelectric focusing, and stained for catalase activity, at least four catalase isoforms were observed, including one dominant form with an isoelectric point of 6.87. Purified pine catalase is not a glycoprotein and has a ratio of absorbance at 208 nm to absorbance at 405 nm of 1.5. When probed with loblolly pine catalase antibodies, protein blots of cell-free extracts from megagametophytes of mature, stratified, and germinated loblolly pine seeds, the megagametophyte glyoxysomal fraction, and purified loblolly pine catalase all revealed one immunoreactive 59-kD polypeptide. This indicates that no detectable change in the enzyme's monomeric molecular mass occurs during seed stratification and germination, early seedling growth, and purification.

Following the completion of germination, oil seeds such as loblolly pine begin to mobilize lipid reserves via the β-oxidation of fatty acids. The subsequent conversion of acetyl-CoA to malate (via the glyoxylate cycle) provides the substrate for carbohydrates that will serve as the primary energy source of the developing young seedling (Beevers, 1979; Huang et al., 1983). Many of the enzymes involved in early seedling metabolism have been studied extensively. In particular, CAT and glyoxylate cycle enzymes such as MS and ICL have all been shown to be highly regulated, undergoing dramatic increases in activity following seed germination and then subsequent declines as lipid reserves are depleted and photosynthetic independence is attained (Beevers, 1979; Huang et al., 1983). Although these enzymes are well characterized in a number of angiosperm systems, an understanding of their developmental regulation in gymnosperms is limited. To date, studies of glyoxylate cycle enzymes and the mobilization of lipid reserves in gymnosperms have been restricted to assessing changes in enzyme activity and quantifying lipid breakdown during and following germination (Ching, 1972; Noland and Murphy, 1984; Pitel and Cheliak, 1986; Groome et al., 1991). In hopes of elucidating some of the developmental controls of gene expression following seed germination and providing some much-needed basic information concerning these processes in gymnosperms, we have begun to examine a number of key enzymes involved in reserve storage mobilization in loblolly pine (*Pinus taeda* L.), including CAT.

CAT (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) has been purified and characterized from various bacterial, fungal, and animal sources (Schonbaum and Chance, 1976) and from a number of angiosperms, e.g. spinach leaves (Galston et al., 1951), cucumber cotyledons (Lamb et al., 1978), maize scutella (Chandlee et al., 1983), pumpkin cotyledons (Yamaguchi et al., 1986), sunflower cotyledons (Eising and Gerhardt, 1986), cotton cotyledons (Kunce et al., 1988), tobacco leaves (Havir and McHale, 1990), and castor bean endosperm (Ota et al., 1992). However, to our knowledge, the enzyme has never been purified from any gymnosperm source. CAT has been consistently characterized as a heme-containing, tetrameric enzyme that is exclusively localized in microbodies (e.g. glyoxysomes and leaf peroxisomes). In glyoxysomes, CAT catalyzes the decomposition of H_2O_2 produced from the β-oxidation of fatty acids (Beevers, 1979; Huang et al., 1983).

CAT has been reported to exist in multiple forms in many higher plants, e.g. spinach (Galston, 1955), mustard (Drumm and Shopfer, 1974), wheat (Singh and Singh, 1975), cotton (Kunce and Trelease, 1986), sunflower (Eising and Gerhardt, 1986), maize (Scandalios, 1987), tobacco (Havir and McHale, 1989), and castor bean (Ota et al., 1992). However, only in cotton and maize have the biochemical and molecular aspects of CAT multiplicity been studied thoroughly (Scandalios, 1987; Ni and Trelease, 1991).

In hopes of understanding the mechanism of the multiplicity of the CAT isoform family in a gymnosperm, we have purified and characterized the enzyme from germinated loblolly pine (*Pinus taeda* L.) megagametophytes. Using a variety of chromatographic techniques, we have separated CAT from

[1] Financial support for this research was provided from Natural Sciences and Engineering Research Council of Canada grant No. OGP0002240 to D.J.G.

* Corresponding author; fax 1–403–492–9457.

Abbreviations: CAT, catalase; DAI, days after imbibition at 30°C; HTP, hydroxylapatite; ICL, isocitrate lyase; MS, malate synthase; pI, isoelectric point.

MS and ICL, both of which have been shown to be frequently contaminated by CAT in the final preparations of these enzymes from other plant sources (Servettaz et al., 1973; Koller and Kindl, 1977; Frevert and Kindl, 1978; Lamb et al., 1978; Pinzauti et al., 1986). On the basis of protein immunoblotting, polyclonal antibodies raised against purified CAT show monospecificity to all forms of the enzyme in loblolly pine megagametophytes, including those from mature, stratified, and germinated seeds, as well as isolated glyoxysomal preparations.

MATERIALS AND METHODS

Seed Material and Germination

Loblolly pine (*Pinus taeda* L.) seeds were kindly provided by Westvaco (Summerville, SC). Lodgepole pine (*Pinus contorta* Dougl), white spruce (*Picea glauca* [Moench] Voss), and Douglas fir (*Pseudotsuga menzieseii* [Mirb] Franco) seeds were all obtained from the Pine Ridge Forest Nursery (Smokey Lake, Alberta, Canada). All seeds were sterilized as previously described (Groome et al., 1991), with the exception that H_2O_2 was omitted. The seeds were then allowed to imbibe on sterile moist Kimpak (Seedboro Equipment, Chicago, IL) at 2°C in the dark for at least 30 d to overcome dormancy. After chilling, the nondormant stratified seeds were surface sterilized in 1% (v/v) NaOCl for 5 min and then transferred to autoclaved Kimpak-lined germination trays. The trays were then placed in a germinator, continuously illuminated at 19 μmol m^{-2} s^{-1} and 30°C (Controlled Environments, Winnipeg, Manitoba, Canada) for up to 10 d, and megagametophytes and developing seedlings were isolated as previously described (Groome et al., 1991). Castor bean (*Ricinus communis* L. cv Hale) seeds were obtained commercially from Bothwell Enterprises (Plainview, TX), and cotton (*Gossypium hirsutum* L. cv Deltapine 62) seeds were kindly provided by Dr. R.N. Trelease (Department of Botany and Microbiology, Arizona State University, Tempe, AZ).

Enzyme and Protein Assays

CAT activity was assayed by the method of Lück (1963). The first-order decomposition of H_2O_2 was measured at 240 nm, and controls contained no H_2O_2. One unit of CAT activity (kunit) was defined as that amount of enzyme that catalyzed the conversion of 1 mmol of H_2O_2 min^{-1} at 22°C. ICL activity was assayed according to the method of Lamb et al. (1978). The formation of glyoxylate phenylhydrazone was monitored as the change in A_{324} using controls containing no isocitric acid. One unit of ICL activity was defined as the amount of enzyme required to degrade 1 μmol of D,L-isocitrate to glyoxylate and succinate per min at 22°C. MS activity was assayed as described by Cooper and Beevers (1969). MS activity was monitored as a change in A_{412}, and controls contained no sodium glyoxylate. One unit of MS activity was defined as the amount of enzyme that released 1 nmol of CoA min^{-1} at 22°C.

All protein extractions for SDS-PAGE were carried out by homogenization in a cold mortar with 50 mM $NaPO_4$, pH 7.5, containing 10 mM DTT and 0.1 mM leupeptin. Protein quantification was by the method of Lowry et al. (1951), with BSA as a standard. The protein concentration of column effluents was monitored spectrophotometrically at 280 nm.

CAT Purification

All procedures were carried out at 4°C. All buffers contained 10 mM DTT and 1 μM leupeptin and were deaerated overnight before use. The following buffers were used: buffer A, 10 mM potassium phosphate (pH 7.0); buffer B, 50 mM Tris-HCl (pH 7.0), 100 mM NaCl; buffer C, 200 mM potassium phosphate (pH 7.0); buffer D, 10 mM Tris-HCl (pH 8.0), 5% (v/v) ethylene glycol.

Isolated frozen megagametophytes (20 g) were ground to a powder in a cold mortar with liquid nitrogen and reextracted with cold acetone (−20°C). The homogenate was centrifuged at 25,000*g* for 20 min, and the resulting precipitate was resuspended in buffer A. After the suspension was homogenized in a Brinkman Polytron blender at high speed, the slurry was filtered through Miracloth and centrifuged at 25,000*g* for 30 min. The supernatant fluid was then fractionated at 4°C by the slow addition of solid $(NH_4)_2SO_4$. Proteins precipitating in the 30 to 70% saturation fraction were resuspended in buffer A and dialyzed overnight against three changes of the same buffer. Insoluble materials were removed by centrifugation at 14,000*g* for 10 min, and the dialyzed sample was applied to a Whatman DE-52 cellulose column (1.5 × 25 cm) previously equilibrated with buffer A. After the column was washed with five column volumes of the same buffer, a 100-mL linear gradient of NaCl (0.0–0.25 M in buffer A) was applied at a flow rate of 30 mL h^{-1}. Peak CAT-activity fractions were pooled and concentrated by $(NH_4)_2SO_4$ precipitation at 4°C (80% saturation). The precipitate was resuspended in 2.5 mL of buffer B and applied directly to a Superdex-200 gel filtration column (3 mL min^{-1}; Pharmacia fast-protein liquid chromatography system) that had been equilibrated in buffer B. Peak CAT-activity fractions were pooled and precipitated with $(NH_4)_2SO_4$ at 4°C (80% saturation), and the resulting pellet was resuspended in 1.2 mL of buffer A. The sample was dialyzed overnight against three changes of buffer A, centrifuged, and loaded directly onto a Bio-Gel HTP column (1 × 12 cm; Bio-Rad) equilibrated in buffer A. The column was washed with 2 column volumes of buffer A and then eluted with a 100-mL linear gradient of 0.01 to 0.2 M potassium phosphate, pH 7.0, at a flow rate of 20 mL h^{-1}. Peak CAT-activity fractions were pooled and concentrated by $(NH_4)_2SO_4$ at 4°C (100% saturation). The resulting pellet was resuspended in 1.5 mL of buffer C and loaded onto a phenyl-Sepharose CL-4B column (1 × 15 cm; Sigma Chemical Co.) previously equilibrated with the same buffer. After the columns were washed with 4 column volumes of buffer C, CAT was eluted with a 100-mL reverse linear gradient (20 mL h^{-1}) consisting of 0.2 to 0 M potassium phosphate, pH 7.0, and 0 to 100% (v/v) ethylene glycol as described by Kunce et al. (1988). Peak CAT-activity fractions were pooled, concentrated to 0.5 mL against buffer D using Centricon-30 Microconcentrators according to the manufacturer's instructions (Amicon Corp.), and stored at −70°C.

Antibody Production

Polyclonal antibodies to CAT were elicited by injecting 30 μg of purified enzyme in 0.75 mL of PBS and 0.75 mL of Freund's complete adjuvant subscapularly and submuscularly at multiple sites of female Flemish giant French lop rabbits. Two separate booster injections were administered every 28 d according to the same injection routes as the initial injections, except 20 μg of CAT protein in 0.75 mL of PBS and 0.75 mL of Freund's incomplete adjuvant was used. Rabbits were bled 8 to 10 d after every booster injection to test for titer. Blood was drawn, incubated at 37°C for 2 h and then 4°C overnight, and centrifuged in a clinical swinging bucket rotor, and the serum was stored at −70°C.

SDS-PAGE

Sample preparation and SDS-PAGE were carried out as described by Laemmli (1970) using a Mini Gel System (Bio-Rad) with a 0.75-mm 12% slab gel at 200 V. Following electrophoresis, proteins were visualized by either Coomassie blue R as described by Burk et al. (1983) or silver stained with a Bio-Rad silver stain kit according to the manufacturer's recommendations (Bio-Rad). The molecular masses of the proteins were determined by the method of Weber and Osborne (1969). Molecular mass markers included: phosphorylase b, 97.4 kD; BSA, 66.2 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD; and lysozyme, 14.4 kD (Bio-Rad).

IEF

Nondenaturing IEF was carried out on a Multiphor II horizontal electrophoresis system (Pharmacia LKB). Gels were cast using a Mini Casting Tray (Bio-Rad) and consisted of 5% polyacrylamide (24.25:0.75 [w/v], acrylamide:bis-acrylamide), 5% (v/v) glycerol, and 5% (v/v) Pharmalyte (pH 5–8). Both the cathodic (20 mM NaOH) and anodic (50 mM acetic acid) solutions were freshly prepared and thoroughly deaerated before use. Samples for IEF were prepared according to the procedures described by Kunce and Trelease (1986) with modifications. Twelve isolated megagametophytes (0.15 g), frozen in liquid nitrogen, were ground to a powder in a cold mortar. Samples were then extracted by continuing grinding in 0.25 mL of 100 mM Tris-HCl (pH 7.0), 20% (v/v) glycerol, 0.1 mM leupeptin, and 20 mM DTT. After centrifugation at 14,000*g* for 20 min, the supernatant cell-free extracts were then diluted in extraction buffer to contain equal amounts of CAT activity. Samples were further diluted (1:1, v/v) with a solution of 80% (v/v) glycerol and 4% (v/v) Pharmalytes (pH 5–8). Electrophoresis was performed at 4°C and 500 V for 15 min, followed by 750 V for 2 h. After focusing, gels were negatively stained for CAT activity using 3,3′-diaminobenzidine as described by Clare et al. (1984) and photographed. In this paper, CAT isoforms are presented as positive images obtained by contact printing using the original gel as a negative. This results in improved visual resolution provided by dark bands on a light background. A Multiphor surface electrode (Pharmacia LKB) was used to determine the pIs of the CAT isoforms.

Glyoxysome Isolation

Glyoxysomes were prepared from loblolly megagametophytes by differential centrifugation according to the procedure described by Maeshima and Beevers (1985). Glyoxysomes were broken osmotically by diluting with 2 volumes of 1 M KCl/1 mM EDTA and were identified by assaying for ICL and MS activity as marker enzymes. Selected glyoxysomal fractions containing peak CAT activity were pooled and prepared for SDS-PAGE analysis as previously described.

Western Transfer and Immunotitration

Following SDS-PAGE, gels were equilibrated for 20 min in transfer buffer (25 mM Tris, 192 mM Gly and 20% [v/v] methanol). The subsequent transfer of proteins to nitrocellulose (Bio-Rad) was carried out overnight in a Mini Trans-Blot Cell (Bio-Rad) according to the manufacturer's recommendations. The nitrocellulose blots were then stained according to the procedures described by Kunce and Trelease (1986). Immunoreactive polypeptides were visualized using alkaline phosphatase activity following the supplier's instructions (Bio-Rad). Immunotitration was carried out as described by Trelease et al. (1987), except samples were prepared in 50 mM $NaPO_4$ (pH 7.5), and 0.10 mM leupeptin.

RESULTS AND DISCUSSION

Purification of CAT

The purification procedure for CAT from isolated loblolly pine megagametophytes (7 DAI) is summarized in Table I and Figure 1. Although consisting of six main steps, including four chromatographic separations, the procedure was carried out in less than 2 d. Following $(NH_4)_2SO_4$ fractionation and

Table I. *Purification of CAT from 20 g of loblolly megagametophytes 7 DAI*

Purification Step	CAT Activity	Protein	Specific Activity	Yield	Purification
	total kunits	*mg*	*kunits/mg*	%	*-fold*
1. Acetone extract homogenate	6690	425.0	16	100	1.0
2. 30–70% saturated ammonium sulfate[a]	3106	83.4	37	46	2.3
3. DE-52 cellulose	1703	16.6	103	25	6.4
4. Superdex-200[a]	1225	2.0	613	18	38.3
5. HTP	800	0.5	1600	12	100
6. Phenyl-Sepharose CL-4B	443	0.2	2215	7	138

[a] CAT activity was assayed after $(NH_4)_2SO_4$ precipitation and overnight dialysis.

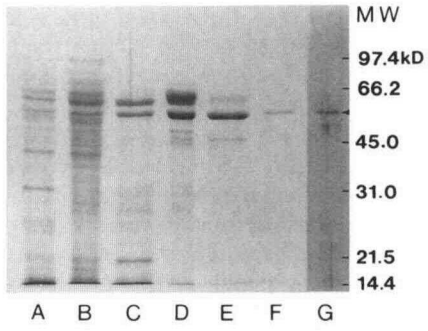

Figure 1. Progress of purification of loblolly pine CAT. Pooled fractions from each stage of the purification procedure (Table I) were subjected to SDS-PAGE on 12% gels and visualized by Coomassie blue staining (A–F). A, Acetone extract (crude homogenate); B, 30 to 70% saturation $(NH_4)_2SO_4$ fraction; C, pooled active fractions from DE-52 cellulose; D, pooled fractions from gel filtration on Superdex-200; E, pooled high CAT/low ICL activity fractions from HTP; F, pooled purified CAT fractions from phenyl-Sepharose CL-4B. G, Silver-stained pooled purified CAT (59 kD) fractions from phenyl-Sepharose CL-4B. Protein (15 μg) was applied to each lane, except in lanes F and G where 5 μg was loaded. The arrow indicates the relative mobility of CAT (59 kD). Numerical values to the right of the gel are molecular masses (in kD) of protein standards.

chromatography on DE-52 cellulose and Superdex-200, initial CAT preparations consistently revealed two dominant protein bands on SDS-PAGE (Fig. 1, lane D). Because of numerous reports identifying CAT as a frequent contaminant of ICL and MS preparations from many higher plants (Servettaz et al., 1973; Koller and Kindl, 1977; Frevert and Kindl, 1978; Lamb et al., 1978; Pinzauti et al., 1986), we examined both enzymes throughout our CAT purification procedure. Despite very low activity due to suboptimal extraction conditions, MS co-purified with CAT during the first four steps of the purification. MS was then completely removed from CAT on HTP, with the former not binding to the matrix and eluting off with the flow-through (data not shown).

At this stage, cotton CAT and ICL antibodies identified loblolly pine CAT as a 59-kD protein and ICL as a 64-kD contaminant (data not shown). Having completely removed MS, HTP chromatography also provided the first step at which CAT could be partially resolved from ICL (Fig. 1, lane E). Because just those fractions containing high CAT activity and low ICL activity were collected, only a 12% overall yield after HTP was achieved. However, this step still resulted in a substantial increase in CAT specific activity, purifying the protein about 2.6-fold (Table I). Despite varying numerous chromatographic conditions, including elution gradients and flow rate, all attempts to separate the enzymes further using HTP chromatography were unsuccessful. In relation to this, Lamb et al. (1978) reported that cucumber CAT and ICL appeared quite similar in chemical and physical properties.

The final separation of loblolly pine CAT from ICL was accomplished by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Fig. 1, lane F). The ability of a hydrophobic matrix to effectively resolve both enzymes has been demonstrated before (Pinzauti et al., 1986). Similar to those findings, and in agreement with ICL's reportedly high hydrophobicity (Vanni et al., 1990), loblolly pine CAT did not bind as strongly to phenyl-Sepharose as did ICL, resulting in their complete resolution. CAT activity eluted from the column at approximately 45% (v/v) ethylene glycol, 0.11 M potassium phosphate, and those active fractions with no detectable ICL activity were pooled, concentrated by ultrafiltration, and stored for antibody production. The final specific activity of the purified CAT preparation was about 2215 kunits mg^{-1}, representing an overall purification of about 140-fold (Table I). Although somewhat higher than most of the published values for the enzyme isolated to date, the final specific activity of loblolly pine CAT may be due simply to the relatively rapid, 2-d purification procedure and/or species-specific stability and high activity of the enzyme. Coomassie blue-stained SDS-PAGE of purified CAT revealed a single band of about 59 kD (Fig. 1, lane F), and only when silver stained could a small number of contaminating proteins be observed (Fig. 1, lane G). To ascertain further the purity of the final CAT preparation, no ICL enzyme activity (data not shown) or protein, as demonstrated by immunoblotting with cotton ICL antibodies (Fig. 2, lane C), was detectable. Results presented in this paper and studies of castor bean (Mullen and Gifford, 1991) indicate that ICL and CAT co-purify through nearly all chromatographic and fractionation techniques used. Consequently, there is the need for precautions when assessing the final purity of CAT preparations from higher plant sources.

Properties of Pine CAT

As determined from its mobility during 7 and 12% SDS-PAGE (Fig. 1, lane F), the subunit molecular mass of purified CAT is approximately 59 kD. The native molecular mass of loblolly pine CAT, as estimated by gel filtration on a calibrated Sepharose CL-6B column (Sigma) is 235 kD, indicating that the enzyme, like all CATs examined to date, is tetrameric.

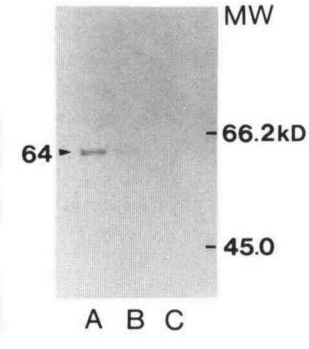

Figure 2. Protein immunoblot demonstrating the purification of loblolly CAT from ICL. Cell-free extract from megagametophytes 7 DAI (A), pooled fractions after HTP (B), and purified CAT (C) were subjected to SDS-PAGE, electroblotted to nitrocellulose, and probed with cotton ICL antibodies. All lanes contain equal amounts of protein (5 μg/lane). A 64-kD ICL band is present in A and is less evident in B but was never detected in purified CAT even when gels were overloaded (data not shown). Numerical values to the right and left of the gel are molecular masses (in kD) of protein standards.

Purified CAT did not react with Schiff's reagent (Zacharius et al., 1969), indicating that it is not a glycoprotein (data not shown). Consistent with most known plant CATs (Eising and Gerhardt, 1986; Yamaguchi et al., 1986), the A_{208}/A_{405} ratio for the purified loblolly pine enzyme was 1.5 (data not shown). This value is consistent with the presence of three to four heme groups per enzyme molecule (Eising and Gerhardt, 1986).

The presence of multiple isoforms of CAT has been reported for a number of plant sources. To identify and characterize CAT isoforms in loblolly pine, cell-free extracts of megagametophytes taken 7 DAI (peak cell-free extract CAT activity; data not shown) were subjected to nondenaturing IEF (Fig. 3A). When gels were stained for CAT activity, four separate isoforms were consistently visualized, including one dominant form (isoform C). On some gels, two additional isoforms were seen (isoforms A and F), but because of their low activity they were difficult to resolve when photographed. When compared to that of cotton cotyledon extracts, the electrophoretic pattern of CAT isoforms from loblolly pine appears quite different (Fig. 3), suggesting that the CAT-isoforms families from both organisms are distinct.

Following a number of precautions outlined by other laboratories interested in CAT multiplicity (Morikoffer-Zwez et al., 1969; Holmes and Masters, 1972; Kunce and Trelease, 1986), we have demonstrated that the electrophoretic pattern of loblolly pine CAT isoforms is not the result of limited proteolysis, enzyme aging, or electrophoretic conditions. The same pattern was observed when extractions included either 20 mM 2-mercaptoethanol or 100 mM EDTA, when gels were pre-electrophoresed, or when protease inhibitors such as leupeptin (1 mM), PMSF (2 mM), and/or benzamidine-HCL

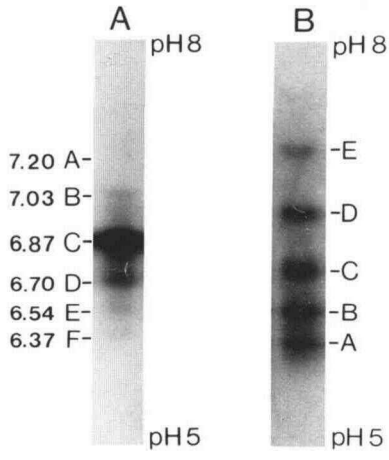

Figure 3. Electrophoretic patterns of CAT isoforms on IEF. Cell-free extracts prepared from loblolly pine megagametophytes 7 DAI (A) and dark-grown cotton cotyledons 4 DAI (B) were subjected to nondenaturing IEF and stained for CAT activity. Because of printing, isoforms A and F from loblolly pine megagametophytes were observed only in the original gel (see lane A). CAT isoforms from cotton (A–E) are those described by Kunce and Trelease (1986). All samples contained 20 mM DTT and were electrophoresed immediately after extraction. The pI for each isoform of loblolly pine is indicated to the left of A.

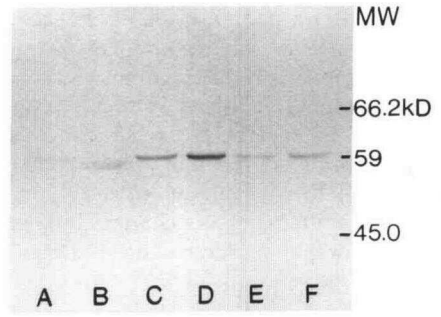

Figure 4. Comparison of immunoblots of CAT extracted from various gymnosperm and angiosperm seeds. Cell-free extracts from castor bean endosperm (4 DAI; A), cotton cotyledons (4 DAI; B), and isolated megagametophytes 7 DAI from Douglas fir (C), loblolly pine (D), lodgepole pine (E), and white spruce (F) were separated by SDS-PAGE, electroblotted, and probed with loblolly pine CAT antibodies. All lanes contained 20 μg of protein. Numerical values to the right of the blot are molecular masses (in kD) of protein standards.

(1 mM) were included (data not shown). In fact, the only time aberrations in the CAT isoform pattern were observed was when extractions were not carried out in the presence of DTT. Kunce and Trelease (1986) previously described the importance of the inclusion of DTT in all extraction buffers when examining the heterogeneity of CAT in cotton.

When examined through a number of developmental stages including poststratification, germination, and postgermative growth, isoform C (Fig. 3A, pI 6.87) was consistently the major CAT form in the megagametophyte (data not shown). Because only one band is detected on SDS-PAGE either by staining of purified CAT with Coomassie blue (Fig.1, lane F) or by probing with cotton or loblolly pine CAT antibodies following immunoblotting of crude or purified samples (data not shown), we believe the CAT isoforms differ on the basis of charge.

Immunological Analysis

To demonstrate the monospecificity of loblolly pine CAT antibodies, the separation of CAT from contaminating ICL during the purification procedure was examined. Protein immunoblots comparing cotton and pine anti-CAT immunoreactive polypeptides were analyzed (data not shown). Even when gels were greatly overloaded or when serum was diluted to 1:50 (as opposed to 1:1000), the 64-kD band (ICL) that reacted when probed with cotton ICL antibodies was not revealed on an immunoblot of the final CAT preparation (Fig. 2). However, both loblolly pine and cotton CAT antibodies reacted with the same single band, migrating and corresponding to the monomeric form of purified loblolly pine CAT (59 kD) (data not shown). Similarly, the fidelity of loblolly pine CAT antibodies was demonstrated by the serum's ability to effectively immunotitrate all CAT activity from megagametophyte extracts 7 DAI. However, neither potential contaminant during the purification procedure (i.e. ICL and MS) was affected by the addition of CAT antiserum (data not shown). Loblolly pine CAT antibodies cross-reacted with

a single polypeptide on immunoblots of cell-free extracts of megagametophytes taken from germinated seeds of white spruce, lodgepole pine, and Douglas fir and two angiosperms, castor bean and cotton (Fig. 4). The subunit molecular mass of cotton CAT was 57 kD (Fig. 4B), whereas it was 59 kD for all other species examined, including castor bean (Fig. 4).

To determine whether the subunit molecular mass of CAT from loblolly pine underwent any changes following mature seed imbibition, megagametophytes and seedlings were taken at various stages, and immunoblots were probed with loblolly pine CAT antibodies (Fig. 5). Cell-free extracts from megagametophytes isolated from mature seeds, stratified seeds, germinated seeds (7 DAI), and purified CAT fractions all reacted with CAT antibodies, and a single, 59-kD band appeared on all blots, indicating that at no time during stratification and postgerminative growth, or during purification, was the monomeric molecular mass of CAT altered. Loblolly pine glyoxysomal CAT is the same size as that from cell-free extracts (59 kD), indicating that no artifactual degradation of CAT occurs by endogenous proteases following its extraction and purification. It remains to be determined whether CAT undergoes any posttranslational modification via proteolytic cleavage of a signal peptide during glyoxysomal import. Yamaguchi et al. (1986) and Kunce et al. (1988) both demonstrated the existence of only one CAT polypeptide from cell-free extracts and isolated glyoxysomes and peroxisomes from pumpkin and cotton cotyledons, respectively. With the apparent consistencies between loblolly pine and angiosperm CAT as already discussed in this paper, it is likely that similar precursor processing, or lack of, might be occurring in loblolly pine.

Despite consistencies in CAT monomeric molecular mass, it remains to be determined whether CAT isoforms undergo any transitions during postgerminative growth. In fact, the availability of monospecific antibodies to CAT will allow us to examine and compare the enzyme in both the haploid maternal megagametophyte and the diploid embryo. In the former, after storage reserves are utilized, the tissue will eventually senesce, whereas embryonic tissue undergoing a change from heterotrophy to autotrophy will display an accompanying glyoxysome to peroxisome transition (Lopez-Perez et al., 1974; Huang et al., 1983). This point is especially interesting when considering the observation that the same 59-kD polypeptide from isolated seedling extracts was recognized by the megagametophytic CAT antibodies (Fig. 5, lane A), suggesting that the CAT families of these two distinct tissue types may be quite similar.

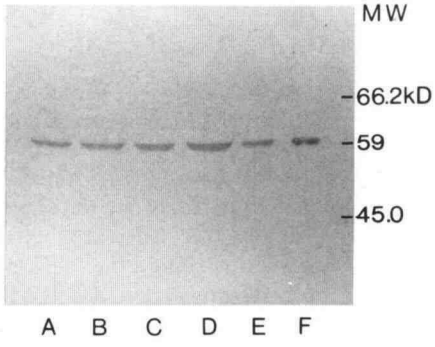

Figure 5. Comparison of loblolly pine CAT following immunoblotting. Protein extracts were separated by SDS-PAGE, electroblotted to nitrocellulose, and probed with loblolly pine CAT antibodies. Extracts were from seedling cotyledons 7 DAI (A), mature seed megagametophytes (B), stratified seed megagametophytes (C), seed megagametophytes 7 DAI (D), purified CAT (E), and glyoxysomes isolated from megagametophytes 7 DAI (F). Protein (15 μg) was applied to each lane (A–E), except in lane F where 5 μg was loaded. Numerical values to the right of the blot are molecular masses (in kD) of protein standards.

ACKNOWLEDGMENTS

We are grateful to Dr. R.N. Trelease for his advice concerning CAT isoform experiments, as well as the generous gift of cotton CAT and ICL antibodies, and Dr. A. Good for assistance with protein purification, use of the fast-protein liquid chromatography system, and stimulating discussions during the course of this work.

Received April 13, 1993; accepted June 8, 1993.

LITERATURE CITED

Beevers H (1979) Microbodies in higher plants. Annu Rev Plant Physiol **30:** 159–193

Burk RR, Eschenbruch M, Leuthard P, Steck G (1983) Sensitive detection of proteins and peptides in polyacrylamide gels after formaldehyde fixation. Methods Enzymol **91:** 247–254

Chandlee JM, Tsaftaris AS, Scandalios JG (1983) Purification and partial characterization of three genetically defined catalases of maize. Plant Sci Lett **29:** 117–131

Ching TM (1972) Metabolism of germinating seeds. *In* TT Kozlowski, ed, Seed Biology, Vol II. Academic Press, New York, pp 103–218

Clare DA, Duong MN, Darr D, Archibald F, Fridovich I (1984) Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and an activity stain for catalase. Anal Biochem **140:** 532–537

Cooper TG, Beevers H (1969) Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. J Biol Chem **244:** 3507–3513

Drumm H, Shopfer P (1974) Effect of phytochrome on development of catalase activity and isoenzyme pattern in mustard (*Sinapis alba* L.) seedlings. Planta **120:** 13–20

Eising R, Gerhardt B (1986) Activity and hematin content of catalase from greening sunflower cotyledons. Phytochemistry **25:** 27–31

Frevert J, Kindl H (1978) Plant microbody proteins. Purification and glycoprotein nature of glyoxysomal ICL from cucumber cotyledons. Eur J Biochem **92:** 35–43

Galston AW (1955) Plant catalase. Methods Enzymol **2:** 789–791

Galston AW, Bommichsen RT, Arnon DI (1951) The preparation of a highly purified spinach leaf catalase. Acta Chem Scand **5:** 781–790

Groome MC, Axler SR, Gifford DJ (1991) Hydrolysis of lipid and protein reserves in loblolly pine seeds in relation to protein electrophoretic patterns following imbibition. Physiol Plant **83:** 99–106

Havir EA, McHale NA (1989) Regulation of catalase activity in leaves of *Nicotiana sylvestris* by high CO_2. Plant Physiol **89:** 952–957

Havir EA, McHale NA (1990) Purification and characterization of an isozyme of catalase with enhanced-peroxidatic activity from leaves of *Nicotiana sylvestris*. Arch Biochem Biophys **283:** 491–495

Holmes RS, Masters CJ (1972) Species specific features of the distribution and multiplicity of mammalian liver catalase. FEBS Lett **11:** 45–48

Huang AHC, Trelease RN, Moore TS Jr (1983) Plant Peroxisomes. Academic Press, New York

Koller W, Kindl H (1977) Glyoxylate cycle enzymes of the glyoxysomal membrane from cucumber cotyledons. Arch Biochem Biophys **181:** 236–248

Kunce CM, Trelease RN (1986) Heterogeneity of catalase in maturing and germinated cotton seeds. Plant Physiol **81:** 1134–1139

Kunce CM, Trelease RN, Turley RB (1988) Purification and biosynthesis of cottonseed (*Gossypium hirsutum* L.) catalase. Biochem J **251:** 147–155

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lamb JE, Riezman H, Becker WM, Leaver CJ (1978) Regulation of glyoxysomal enzymes during germination of cucumber. 2. Isolation and immunological detection of ICL and catalase. Plant Physiol **62:** 754–760

Lopez-Perez MJ, Gimenez-Solves A, Calonge FD, Santos-Ruiz A (1974) Evidence of glyoxysomes in germinating pine seeds. Plant Sci Lett **2:** 377–386

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Lück H (1963) Catalase. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis. Academic Press, New York, pp 886–894

Maeshima M, Beevers H (1985) Purification and properties of glyoxysomal lipase from castor bean. Plant Physiol **79:** 489–493

Morikoffer-Zwez S, Cartz M, Kaufmann H, Von Wartbug JP, Aebi H (1969) Heterogeneity of erythrocyte catalase. Correlations between sulfhydryl group content, chromatographic and electrophoretic properties. Eur J Biochem **11:** 49–57

Mullen RT, Gifford DJ (1991) Effects of the embryo axis on catalase gene expression in the endosperm of castor bean following imbibition. *In* RB Hallick, ed, Proceedings of the 3rd International Congress of the International Society of Plant Molecular Biology, Abstract 646

Ni W, Trelease RN (1991) Post-transcriptional regulation of catalase isozyme expression in cotton seeds. Plant Cell **3:** 737–744

Noland TL, Murphy JB (1984) Changes in isocitrate lyase activity and ATP content during stratification and germination of sugar pine seeds. Seed Sci Technol **12:** 777–787

Ota Y, Ario T, Hayashi K, Nakagawa T, Hattori T, Maeshima M, Asahi T (1992) Tissue-specific isoforms of catalase subunits in castor bean seedlings. Plant Cell Physiol **33:** 225–232

Pinzauti G, Giachetti E, Camici G, Manao G, Cappugi G, Vanni P (1986) An isocitrate lyase of higher plants: analysis and comparison of some molecular properties. Arch Biochem Biophys **244:** 85–93

Pitel JA, Cheliak WM (1986) Enzyme activities during imbibition and germination of seeds of tamarack (*Larix larienina*). Physiol Plant **67:** 562–569

Scandalios JG (1987) The antioxidant enzyme genes *Catalase* and *Sod* of maize: regulation, functional significance and molecular biology. Isozymes Curr Top Biol Med Res **14:** 19–44

Schonbaum GP, Chance B (1976) Catalase. *In* PD Boyer, ed, The Enzymes, Ed 3, Vol 13. Academic Press, New York, pp 363–408

Servettaz O, Filippini M, Longo CP (1973) Purification and properties of malate synthase from maize scuttela. Plant Sci Lett **1:** 71–80

Singh R, Singh D (1975) Peroxidase, polyphenol oxidase and catalase isoenzymes during germination and early plant development of tall and dwarf wheats (*Triticum aestivum* L.). Biol Plant **17:** 235–240

Trelease RN, Hermerath CA, Turley RB, Kunce CM (1987) Cotton seed malate synthase. 1. Purification and immunochemical characterization. Plant Physiol **84:** 1343–1349

Vanni P, Giachetti E, Pinzauti G, McFadden B (1990) Comparative structure, function and regulation of isocitrate lyase, an important assimilatory enzyme. Comp Biochem Physiol **95:** 431–458

Weber K, Osborne M (1969) The reliability of molecular weight determinations by sodium dodecyl sulfate polyacrylamide gel electrophoresis. J Biol Chem **244:** 4406–4412

Yamaguchi J, Nishimura M, Akazawa T (1986) Purification and characterization of a heme-containing low-activity form of catalase from greening pumpkin cotyledons. Eur J Biochem **159:** 315–322

Zacharius RM, Zell TE, Morrison JH, Woodlock JJ (1969) Glycoprotein staining following electrophoresis on acrylamide gels. Anal Biochem **30:** 148–152