Maturation of catalase precursor proceeds to a different extent in glyoxysomes and leaf peroxisomes of pumpkin cotyledons*

(inactive enzyme/microbody transition/protein synthesis)

JUNJI YAMAGUCHI, MIKIO NISHIMURA, AND TAKASHI AKAZAWA

Research Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

Communicated by Elizabeth F. Neufeld, March 29, 1984

ABSTRACT As an approach to study the mechanism of the microbody transition (glyoxysomes to leaf peroxisomes) in greening pumpkin cotyledons, catalase molecules were purified from the two different types of microbody and their structural properties were compared. The purified glyoxysomal catalase was found to consist of four identical subunits (55 kDa), whereas the leaf peroxisomal catalase contains two different forms of monomeric subunit (55 and 59 kDa). These different catalase species cross-reacted with the rabbit antibody raised against the glyoxysomal enzyme. During gel filtration on an Ultrogel AcA 34 column, the leaf peroxisomal 55-kDa polypeptide eluted slightly faster than the leaf peroxisomal 59-kDa polypeptide. The profile of catalase activities exactly paralleled the elution pattern of the 55-kDa molecules, which indicated that the 59-kDa polypeptide was enzymatically inactive. Peptide mapping analysis using Staphylococcus aureus protease V8 showed that the glyoxysomal 55-kDa polypeptide was identical to the leaf peroxisomal 55-kDa species, whereas the leaf peroxisomal 59-kDa polypeptide had a different primary structure from the 55-kDa polypeptide. In an in vitro translation system directed by mRNA isolated from etiolated and green cotyledons, glyoxysomal and leaf peroxisomal catalases were synthesized as the identical 59-kDa polypeptide. From peptide mapping analysis, the in vitro-translated 59-kDa polypeptide was found to have a nearly identical primary structure to that of the leaf peroxisomal 59-kDa species. In vivo pulse-chase labeling experiments using etiolated cotyledons showed the conversion of the 59-kDa polypeptide to the 55-kDa molecular species. The overall results strongly indicate that the 59-kDa polypeptide is a precursor form of catalase in pumpkin cotyledons.

In the cotyledons of some fatty seeds such as pumpkin and watermelon, there occurs the transitional formation of leaf peroxisomes from glyoxysomes during greening (1). During the step, glyoxysomal enzyme activities are known to be selectively lowered, and concomitantly, leaf peroxisomal enzyme activities are prominently enhanced (2, 3). Studies on catalase are considered to provide unique information to clarify the mechanism of the microbody transition, because the enzyme is localized in both glyoxysomes and leaf peroxisomes (4). A question pertaining to this problem is whether there exist any structural or functional differences between glyoxysomal and leaf peroxisomal catalase molecules.

Previously we purified glyoxysomal catalase from etiolated pumpkin cotyledons and its immunoochemical properties were compared with the leaf peroxisomal enzyme by using anti-glyoxysomal catalase IgG (5). Pumpkin glyoxysomal catalase was shown to consist of four identical monomeric subunits (55 kDa). Its specific activity is higher than the enzyme from some other plant sources (6-8), and the enzyme protein contains a higher content of heme. Although the glyoxysomal catalase was found to be immunoochemically identical with the leaf peroxisomal enzyme, its specific activity was 2.5-fold higher than that of the leaf peroxisomal enzyme (5). We have shown that reduction of catalase specific activities occurs during the entire step of the microbody transition. Indeed, it is known that total catalase activities in the fatty seedlings decrease during the microbody transition (3, 9).

To elucidate the picture of the microbody transition at the molecular basis, we have purified both glyoxysomal and leaf peroxisomal catalases from pumpkin seedlings and compared their structural properties. Consequently, we have detected an inactive form of the catalase molecule (59 kDa), which is believed to be the precursor of the enzyme protein.

MATERIALS AND METHODS

Plant Growth and Purification of Glyoxysomal and Leaf Peroxisomal Catalase. Pumpkin seeds (Cucurbita sp. Ama-kuri Nankin) were germinated under the conditions described previously (5). Either etiolated cotyledons (170 g) of pumpkin seedlings grown in the dark for 4 days or green cotyledons (300 g) excised from seeds that were grown in the greenhouse for 2 wk were placed in 3 vol of chilled acetone (-10°C) for 2 hr. The tissues were homogenized with a Waring blender at the maximal speed, washed three times with chilled acetone (ca. 2 liters), and then dried to prepare acetone powders. The acetone powder was suspended in 0.1 M Na2HPO4 (2.8 vol per g of fresh weight) and was vigorously stirred for 1 hr. Then the whole slurry was passed through eight layers of cheesecloth and one layer of Miracloth. The extract was centrifuged at 30,000 x g for 20 min, and the clear supernatant fraction obtained was subjected to 20-45% (NH4)2SO4 fractionation. The precipitate was dissolved in a small volume of 0.1 M Na2HPO4 and dialyzed against 5 liters of 10 mM Tris/HCl buffer (pH 8.5) for 12 hr. The conditions for subsequent DEAE-cellulose column chromatography and Ultrogel AcA 34 gel filtration were essentially the same as those described (5).

Enzyme Assay. Catalase activity was measured following the method of Luck (10). Protein content was determined by Lowry's method by using bovine serum albumin as a standard protein (11).

Electrophoretic Transfer Blotting. Electrophoretic blotting was performed basically as described by Towbin et al. (12) and Vaessen et al. (13). NaDodSO4 gel electrophoresis was carried out according to the procedure of Laemmli (14), and the protein components separated were transferred electrophoretically to a nitrocellulose sheet (Toyo TM-2, 0.45-μm pore size). The electrode buffer (pH 8.3) contained 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol. A constant current of 140 mA was applied to complete the transfer during 1.5 hr.

The electrophoretic blots were soaked in a saline solution

*This is paper no. 4 in the series "Analytical Studies on the Microbody Transition" and paper no. 3 is ref. 5.
(0.9% NaCl/0.1% Na2) containing 3% bovine serum albumin for 1 hr; subsequently, they were rinsed in saline solution and incubated overnight with the anti-glyoxysomal catalase serum (0.5 ml), which had been diluted to 110 ml with saline solution containing 3% bovine serum albumin. After washing with the saline solution, the blots were incubated with the fluorescein isothiocyanate-labeled anti-rabbit IgG goat IgG (Cappel Laboratories, Cochranville, PA) to localize the immunoreactive catalase polypeptides, and finally sheets were completely washed with saline solution before taking photographs.

**Peptide Mapping Using Protease V8.** Partial enzymatic proteolysis of the protein samples with *Staphylococcus aureus* protease V8 (Miles) was performed according to the procedure described by Cleveland et al. (15). After the electrophoretic run, the NaDodSO4/polyacrylamide gel containing the catalase molecule (see Fig. 1) was cut, and the segments were put directly on the sample gel containing protease V8. By the rerunning of 55- and 59-kDa species eluted from the gel on gel electrophoresis, it was confirmed that each component is free from cross-contamination.

**Isolation of RNA.** Isolation of RNA from pumpkin cotyledons was performed following the procedures described by Miyata et al. (16). Starting with 70 g each of either etiolated or green pumpkin cotyledons, the tissue was ground to a fine powder in liquid N2 with a mortar and pestle. To the powder was added 200 ml of 50 mM N-2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine/KOH buffer, pH 9.5, containing 150 mM NaCl, 20 mM EDTA, and 2% NaDodSO4. After the addition of an equal volume of phenol/chloroform, 1:1 (vol/vol), to the suspension, RNA was extracted into the aqueous phase by vigorous stirring. The aqueous phase was separated by centrifugation and treated once again with an equal volume of phenol/chloroform (1:1). The RNA was precipitated by the addition of ethanol to the aqueous phase at a final concentration of 70%. The precipitate collected was washed twice with 70% ethanol and twice with 3 M sodium acetate (pH 5.5). The washed precipitate was suspended in sterile distilled H2O and the RNA fraction was precipitated by the addition of LiCl to a final concentration of 2 M in an ice bath. After washing with 70% ethanol, the resulting precipitate dissolved in sterile distilled H2O was used as the mRNA fraction.

**In Vitro Synthesis of Catalase.** RNA fractions isolated from either etiolated or green cotyledons were then used in an *in vitro* translation system containing wheat germ extracts or reticulocyte lysates. In the reticulocyte lysate cell-free system (50 μl) (Radiochemical Centre), 50 μCi (1 Ci = 37 GBq) of [35S]methionine and RNA (0.3 mg/ml) were included in a reaction mixture (total, 61.5 μl). The translation system using wheat germ extracts was essentially the same as that described by Roberts and Paterson (17), in a reaction mixture containing [35S]methionine (50–150 μCi) and RNA (0.3 mg/ml). In both cases, at the end of incubation for 1 hr at 30°C the reaction was stopped by adding NaDodSO4 at a final concentration of 2% (wt/vol) and the mixtures were heated at 80°C for 3 min, followed by the addition of Triton X-100 to 4% (wt/vol). After the addition of 0.5 ml of phosphate-buffered saline (P/NaCl) (pH 7.4), 10–20 μg of anti-glyoxysomal catalase IgG was added and incubation was continued for an additional hour at 30°C. Five milligrams (dry weight) of protein A-Sepharose CL-4B (Sigma) was added to the mixture, which was allowed to incubate for 1 hr at 30°C with occasional stirring. Finally, the mixture was applied to a small column to collect the protein A-Sepharose gel with the bound immunoprecipitates. After thoroughly washing the column with the P/NaCl containing 1% Triton X-100 followed by distilled H2O in a stepwise manner, the immunoprecipitates were eluted with 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% NaDodSO4, 5% 2-mercapto-ethanol, 10% glycerol, and 0.001% bromophenol blue. The eluates were then analyzed by NaDodSO4 gel electrophoresis (14) and fluorography.

**Pulse–Chase Experiments.** [35S]Methionine (100 μCi) was administered to the inner surface of a pair of cotyledons from an etiolated pumpkin seedling (3-day). After 30 min of incubation on a moist filter paper at 25°C, the tissue was washed three times with unlabeled methionine; one half of the cotyledon was homogenized immediately and the other half was homogenized after a 60-min chase with 1 ml of 100% acetone in a mortar and pestle. The pelleted fraction obtained (8000 × g, 5 min) was dissolved in 0.5 ml of 2% NaDodSO4 solution containing 2 mM methionine and boiled for 4 min. The sample was then subjected to immunoprecipitation, NaDodSO4 gel electrophoresis, and fluorography as described above after the addition of Triton X-100 (final concentration, 4%).

**RESULTS**

**Purification of Catalase from Etiolated and Green Pumpkin Cotyledons.** Catalase was purified from etiolated and green cotyledons of pumpkin seedlings under identical conditions. The elution profiles of catalase activities in DEAE-cellulose and Ultrogel AcA 34 column chromatography were identical in both extracts. From the NaDodSO4 gel electrophoresis purified catalase from the etiolated cotyledons was shown to contain only one subunit component of 55 kDa (Fig. 1, lane B), indistinguishable from that in the enzyme molecule prepared from the glyoxysomes (lane A) (5). However, there were two protein bands detectable in the final preparation obtained from the green cotyledons, with sizes of 55 and 59 kDa (lane C).

The immunochromatographic characterization of the catalase molecule employing the electrophoretic transfer blotting technique is presented in Fig. 2. It is clear that the 55- and 59-kDa subunits cross-react with the rabbit antiserum raised against the glyoxysomal 55-kDa catalase. To exclude the possible artifactual formation of the 55-kDa species of the proteolytic digestion of the 59-kDa species, both etiolated and green cotyledons were homogenized with Tris-HCl buffer (pH 6.8) containing 4% NaDodSO4 and 10% mercaptoethanol and the extracts were quickly boiled. The results showed that both the 55- and 59-kDa species were detectable (lane A), indicating that these two molecular species are not artifacts. It can be seen that in the purified glyoxysomes, not only the 55-kDa species but also the 59-kDa species were detected, just as in the leaf peroxisomes (lane C). Although the electrophoretic transfer blotting method itself is supersensitive and fluorescein isothiocyanate labeling does not strictly reflect the amounts of each subunit, it is clear that the 55-kDa species

![](image.png)

**FIG. 1.** NaDodSO4 gel electrophoresis of catalase. Lanes: A, catalase purified from glyoxysomes; B, catalase purified from etiolated cotyledons; C, catalase purified from green cotyledons. Sizes are given in kDa.
Fig. 2. Immunochemical characterization of catalase by the electrophoretic transfer blotting technique. Lanes A, NaDODSO$_4$ extract: etiolated and green cotyledons were homogenized in 125 mM Tris-HCl buffer (pH 6.8) containing 4% NaDODSO$_4$, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue, and the whole extracts were quickly boiled for 3 min. Lanes B, crude homogenate: etiolated and green cotyledons were homogenized by a razor blade chopping method using 150 mM N-(2-hydroxyethyl)-1,1-bis(hydroxymethyl)ethyl]glycine/KOH buffer (pH 7.5) containing 1 mM EDTA and 13% (wt/vol) sucrose. The whole homogenate was used after filtration through four layers of cheesecloth. Lanes C, microbody fraction: crude homogenates (see above) were applied to a sucrose density gradient (30–60%, wt/wt) to separate glyoxysomes and leaf peroxisomes (see refs. 5 and 26). Lanes D, purified catalase from etiolated and green cotyledons (see Fig. 1).

was predominant in the glyoxysomes, whereas the 59-kDa species was predominant in the leaf peroxisomes (lane C). Enzymically Inactive 59-kDa Catalase in Leaf Peroxisomes. As reported previously (5), the specific activity of the glyoxysomal 55-kDa catalase is 90–120 kilounits/mg, whereas that of the leaf peroxisomal origin is lower (20–40 kilounit/mg). Because leaf peroxisomal catalase contains a mixture of 55- and 59-kDa subunits (Fig. 2), we attempted to separate the two catalase species in leaf peroxisomes. Fig. 3 clearly shows that the two species can be resolved by gel filtration, and the elution of enzyme molecules containing the 59-kDa subunit is much retarded relative to that of the 55-kDa species. More important, catalase activity was coincident with the elution profile of enzyme containing the 55-kDa subunit. From further purification of the leaf peroxisomal catalase, the specific activity of the 59-kDa catalase was calculated to be <10 kilounits/mg of protein (data not shown). On the other hand, assuming that the catalase molecule composed of 59-kDa subunits does not have enzyme activity, the specific activity of leaf peroxisomal 55-kDa catalase is calculated to be ~100 kilounits/mg, nearly equivalent to that of the glyoxysomal 55-kDa catalase.

Here, apparent discrepant distribution of the 59- and 55-kDa polypeptides in crude homogenates (Fig. 2, lane B) and purified preparations (Fig. 1, lanes B and C) merits description. The elution profile of the 59-kDa species was different from that of the 55-kDa species in Ultrogel AcA 34 gel filtration (Fig. 3) and DEAE-cellulose column chromatography (data not shown). However, the actual purification was performed by using catalase activity as an index. Therefore, the recovery of the enzymatically active 55-kDa form is inevitably high compared to the inactive 59-kDa species. On the other hand, the 59-kDa species, present only in a small quantity in the etiolated cotyledons, can be effectively removed during the purification. But they are clearly detectable in the final pure preparation obtained, as they are predominantly present in the green tissues.

Peptide Mapping of Three Catalase Species. Three molecular species of catalase—i.e., glyoxysomal (55 kDa), leaf peroxisomal (55 and 59 kDa)—were subjected to limited proteolytic digestion and the peptide fragments were separated electrophoretically (15) (Fig. 4). Digestion by 2 μg of S. aureus protease V8 yielded six identical peptides from both glyoxysomal and leaf peroxisomal 55-kDa species (lanes A and B). On the other hand, in the case of the leaf peroxisomal 55-kDa species, six peptides were shown to be common between 55 and 59 kDa, but at least four additional peptides, not present in the 55-kDa species, were clearly detected (lane C). Similar results were obtained after digestion with 0.2 μg of protease V8 (Fig. 3, left) or when trypsin (1.5 μg) or chymotrypsin (1.5 μg) was used in place of protease V8 (data
authentic glyoxysomal 55-kDa catalase (lane A). Competition experiments, in which an excess of unlabeled glyoxysomal 55-kDa molecules was added to the translation products prior to immunoprecipitation, resulted in the complete disappearance of the radioactive 59-kDa species (lanes B and F). On the basis of these data, we conclude that the translation products directed by mRNA isolated from either etiolated or green cotyledons are the identical molecular entities with sizes of 59 kDa.

As shown in Fig. 5B, the primary structures of the in vitro-synthesized 59-kDa products (green mRNA) (see lane C in Fig. 5A) and the purified leaf peroxisomal 59-kDa molecules (see lane C in Fig. 1) appear to be identical from the pattern of peptide mapping. Gel segments containing each of the 59-kDa catalase species were put on the sample gel supplemented with S. aureus protease V8 at two levels. The fluorographic pattern (lanes B) of the in vitro-synthesized 35S-labeled 59-kDa molecules and the protein bands stained with Coomassie brilliant blue (lanes A) were found to be identical, which indicated that leaf peroxisomal 59-kDa catalase has nearly the identical primary structure as that of the in vitro synthesized leaf peroxisomal catalase.

Employing pulse–chase labeling of etiolated cotyledons (30-min pulse with [35S]methionine and 60-min chase with 10 mM unlabeled methionine), a marked decrease in labeling of the 59-kDa species was found, concomitant with a marked increase in labeling of the 55-kDa catalase molecule (Fig. 5C). The results provide strong evidence that the 59-kDa species is the precursor of the 55-kDa catalase.

**DISCUSSION**

The results obtained in the present investigation are summarized in Table 1, which shows that two types of catalase molecules, composed of 55- and 59-kDa subunits, exist in glyoxysomes and leaf peroxisomes. Concerning the different molecular forms of catalase, Kindl (19) reported the presence of two forms of catalase (53.5 and 57 kDa) in lentil leaves, although the molecular characterization was not performed. Also, the in vitro translation product of rat liver peroxisomal catalase was reported to be about 4000 larger in size than the purified native enzyme molecules, although it was, in fact, found to be ascribable to artifactual modification of the polypeptide during the purification step (20). However, this possibility is ruled out in the present investigation, and it is most likely that the 59-kDa species is the
Table 1. Two molecular forms of catalase in pumpkin cotyledons

<table>
<thead>
<tr>
<th>Cotyledon</th>
<th>Subunit, kDa</th>
<th>Native, kDa</th>
<th>Specific activity, kunit/mg</th>
<th>Localization</th>
<th>In vitro, kDa</th>
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<td>230</td>
<td>90–120</td>
<td>Glyoxysomes</td>
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<td>230</td>
<td>100</td>
<td>Leaf peroxisomes</td>
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<td></td>
<td>59</td>
<td>215*</td>
<td>10</td>
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*Estimation from the elution profile by Ultrogel AAc 34 column chromatography (see Fig. 3).

The precursor form of the 55-kDa catalase in both glyoxysomes and leaf peroxisomes of pumpkin seedlings for the following reasons: (i) The presence of both 59- and 55-kDa catalase in situ was proven by electrophoretic transfer blotting (Fig. 2). (ii) In contrast to the high specific activity of the 55-kDa catalase (~100 kilounit/mg of protein), the 59-kDa catalase isolated from leaf peroxisomes was enzymically inactive. Our results indicate that the molecular entity of the enzymically active form of catalase is the 55-kDa polypeptide (Fig. 3). (iii) The 59-kDa catalase had a similar but different primary structure from that of the 55-kDa catalase (Fig. 4). (iv) It was found that the in vitro translation system of catalase directed by mRNA isolated from both etiolated and green cotyledons synthesized a 59-kDa polypeptide (Fig. 5A). (v) Leaf peroxisomal 59-kDa catalase had nearly the identical primary structure as that of the in vitro synthesized leaf peroxisomal catalase (Fig. 5B). (vi) In vivo pulse-chase [35S]methionine-labeling experiments using the etiolated cotyledons showed the conversion of the 59-kDa polypeptide to the 55-kDa catalase molecule (Fig. 5C).

It now appears evident that both glyoxysomal and leaf peroxisomal catalase molecules are synthesized as an identical polypeptide of 59 kDa and, during the step of microbody biosynthesis, the precursor peptide is transported to each organelle without processing. This mechanism can be supported by the fact that the 59-kDa species are detectable in both glyoxysomes and leaf peroxisomes (Fig. 2). However, it must be stressed that the subsequent maturation step of the enzyme molecule in the pumpkin cotyledons is unique as compared with the biosynthesis of catalase in animal tissues as well as yeast cells (20–23)—that is, in pumpkin, the processing appears to be a prerequisite step in the transformation of the polypeptide into the enzymically active molecular form. In the glyoxysomes, most of the newly synthesized precursor molecules are transformed into the active 55-kDa species, whereas, in the leaf peroxisomes, the transported molecules remain in the inactive form without processing. Therefore, it is surmised that a decline in the catalase activity in cotyledons during the step of greening (see refs. 3, 9) is due to an increase of the enzymically inactive 59-kDa molecules and that the magnitude of the protein processing in the glyoxysomes is greater than that in the leaf peroxisomes, in the latter presumably the process being subject to the regulation by light.

It has been reported that in vitro products of two microbody enzymes—i.e., malate dehydrogenase (18) and thiolase (24)—are larger than the mature molecule, whereas most other microbody enzymes are synthesized in a form that is the same as the mature form (25). In light of our current knowledge concerning the post-translational processing of protein molecules transported into chloroplasts and mitochondria, it is an intriguing question to ask whether or not protein transport into microbodies requires a transit signal peptide. In this report, we have shown that catalase is synthesized as a higher molecular weight precursor form (59 kDa); the latter molecular species are indeed detectable in the organelles, indicating that processing is not required obligatorily for the transport mechanism but is involved in the enzyme activation. It remains open for future investigation to examine whether or not malate dehydrogenase and thiolase entail a similar maturation mechanism as that for catalase.

From the elution profile of Ultrogel AAc 34 presented in Fig. 3, the size of the native form of the 59-kDa catalase can be estimated to be 215 kDa, slightly smaller than the 55-kDa catalase holoenzyme. This result indicates that the 59-kDa catalase also has a tetrameric structure, but the conformation of the holoenzyme may be different from that of 55-kDa species. At the present time, a definite answer cannot be given as to whether there exists a hybrid catalase containing both 55- and 59-kDa molecules or a mixture of homologous enzyme molecules containing each of the 55- and 59-kDa species in microbodies. Further rigorous studies are needed to clarify these unsolved problems, and the structural comparison between the 59- and 55-kDa catalase will eventually lead us to a clue necessary for clarification of the hitherto unknown molecular mechanism of the microbody transition in fatty seedlings.