Plant Physiol. (1984) 74, 261-267
0032-0889/84/74/0261/07/$01.00/0

Purification of Glyoxysomal Catalase and Immunochemical Comparison of Glyoxysomal and Leaf Peroxisomal Catalase in Germinating Pumpkin Cotyledons[1]

Received for publication May 24, 1983 and in revised form August 22, 1983

JUNJI YAMAGUCHI AND MIKIO NISHIMURA*
Research Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

ABSTRACT

As a step to study the mechanism of the microbody transition (glyoxysomes to leaf peroxisomes) in pumpkin (*Cucurbita sp.* Amakuri Nankin) cotyledons, catalase was purified from glyoxysomes. The molecular weight of the purified catalase was determined to be 230,000 to 250,000 daltons. The enzyme was judged to consist of four identical pieces of the monomeric subunit with molecular weight of 55,000 daltons. Absorption spectrum of the catalase molecule gave two major peaks at 280 and 405 nanometers, showing that the pumpkin enzyme contains heme. The ratio of absorption at 405 and 280 nanometers was 1.0, the value being lower than that obtained for catalase from other plant sources. These results indicate that the pumpkin glyoxysomal catalase contains the higher content of heme in comparison with other plant catalase.

The immunochemical resemblance between glyoxysomal and leaf peroxisomal catalase was examined by using the antiserum specific against the purified enzyme preparation from pumpkin glyoxysomes. Ouchterlony double diffusion and immunoelectrophoretic analysis demonstrated that catalase from both types of microbodies cross-reacted completely whereas the immunotitration analysis showed that the specific activity of the glyoxysomal catalase was 2.5-fold higher than that of leaf peroxisomal catalase. Single radial immunodiffusion analysis showed that the specific activity of catalase decreased during the greening of pumpkin cotyledons.

In some seeds such as pumpkin and watermelon, fat is stored as reserve material in the cotyledons. During the course of germination of seeds, this reserve fat is utilized as the primary energy source for the growth of young seedlings. During the period when the cotyledons emerge from the soil, tissues become green and photosynthetically active upon exposure to light. Evidently, photosynthesis supplies the major portion of energy needed for the growth of the seedlings after greening.

During the greening process, several metabolic and structural changes, such as the developmental formation of chloroplasts from plastids and the transitional formation of leaf peroxisomes from glyoxysomes, are known to occur (1, 2). Since there is no cell division in the cotyledons of these fatty seedlings during the process of germination and subsequent greening, all the developmental events take place in a fixed number of pre-existing cells. Therefore, the cotyledons are believed to be a feasible research material for studying the regulatory mechanism underlying the organelle development as well as its transformation.

The transformation of microbodies (glyoxysomes to leaf peroxisomes) in greening fatty seedling is an interesting phenomenon, and two hypothetical mechanisms (the one-population model and the two-population model) have been put forward regarding the origin of the leaf peroxisomes in green tissues. The one-population model postulates that glyoxysomes are considered to be directly transformed to the leaf peroxisomes during the greening process (2, 29). On the other hand, in the two-population model, it is assumed that glyoxysomes are entirely broken down during greening and leaf peroxisomes are newly synthesized *de novo* (1, 13, 14).

Studies on catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) are considered to provide useful opportunities to elucidate the mechanisms of microbody transition, as the enzyme is a well known marker of both glyoxysomes and leaf peroxisomes (11). According to the one population model, it is conceivable that catalase present in glyoxysomes is not broken down, but retained in the leaf peroxisomes. The two-population model predicts that catalase would be completely broken down like other glyoxysomal enzymes, while leaf peroxisomal catalase is newly synthesized. At present it is not known whether there are any differences between glyoxysomal and leaf peroxisomal catalase molecules.

Catalase has been purified from various animal and bacterial sources, but there are only a few reports concerning the purification of catalase from plant sources, *e.g.* spinach leaves (9), lentil leaves (28), cucumber cotyledons (16), and sweet potato roots (7). Emden (6) attempted to purify the enzyme from the pumpkin cotyledons in the late 1940s, but he was unsuccessful because of the lability of the enzyme.

In this communication, we report the purification and the characterization of catalase from glyoxysomes in etiolated pumpkin cotyledons, and immunochemical comparison between the glyoxysomal and the leaf peroxisomal enzyme using the anti-glyoxysomal catalase serum (IgG).[2]

MATERIALS AND METHODS

Plant Growth Conditions. Pumpkin seeds (*Cucurbita sp.* Amakuri Nankin) were soaked in running tap water for 1 d, and seed coats were removed, then germinated on moist filter paper at 25°C in the dark. In order to study catalase during greening, the dark-grown seedlings planted in moist vermiculite were ex-

[1] The research was supported in part by research grants from the Ministry of Education, Science and Culture of Japan (5776065) and the Ishida Foundation (Nagoya). This is Paper 3 in the series "Analytical Studies on Microbody Transition." Paper 2 of the series is Ref. 23.

[2] Abbreviations: IgG, immunoglobulin G; KPB, potassium phosphate buffer.

posed to a continuous illumination. For preparing leaf peroxisomes from green cotyledons, pumpkin seedlings which were grown in moist vermiculite for 10 to 14 d in the greenhouse were used as starting materials. The activity of isocitrate lyase (glyoxysomal marker enzyme) (3) was not detectable in the extracts, indicating that the microbody transition had occurred.

Purification of Glyoxysomal Catalase. *Step 1: Sucrose Density Gradient Centrifugation.* After 4 d of growth in the dark, the etiolated pumpkin cotyledons (110 g) were placed in a Petri dish on an ice bath. The tissue was homogenized in 75 ml of chilled medium containing 150 mM Tricine-KOH (pH 7.5), 1 mM EDTA, and 13% (w/v) sucrose by chopping with a stainless steel razor blade for 20 min, and the step was repeated 2 times. Totally, 330 g of the etiolated cotyledons were homogenized, the whole homogenates were passed through four layer of cheesecloth, and the filtrate was directly layered on top of the sucrose gradient (24); 15 ml was applied to each 25-ml linear sucrose gradient 30 to 60% (w/w) containing 1 mM EDTA (adjusted to pH 7.5). The centrifugation was performed at 21,000 rpm for 90 min at 4°C using a Spinco SW 27 rotor (6 tubes) in Beckman L2-65B ultracentrifuge. The glyoxysomal fraction was collected by a Pasteur pipette. This centrifugation step was repeated 3 times. For the analysis of marker enzyme distribution, 1.2-ml fractions were collected with an ISCO density gradient fractionator (model 640) and used for the assay of each respective marker enzyme.

Step 2:$(NH_4)_2SO_4$ precipitation. The glyoxysomal fraction (82 ml) was dialyzed against 10 mM KPB (pH 8.0) containing 1 mM EDTA (2 L, twice) overnight; during this step, the glyoxysomes were completely disrupted due to the osmotic shock, and the soluble proteins including catalase were separated from the membranous components by centrifugation at 100,000*g* for 1 h. The supernatant fractions were precipitated by the addition of $(NH_4)_2SO_4$ at 70% saturation, and the precipitate was dissolved in 5 ml of 10 mM KPB (pH 8.0) containing 1 mM EDTA.

Step 3: Ultrogel AcA 34 Gel Filtration. The crude enzyme solution was loaded onto an Ultrogel AcA 34 (LKB) column (117 × 1.7 cm) which had been pre-equilibrated with 10 mM KPB (pH 8.0) containing 1 mM EDTA, and eluted by the same buffer at a flow rate of 10 ml/h. The enzymically active fractions were collected and dialyzed against 10 mM Tris-HCl buffer (pH 8.5) for 12 h.

Step 4: DEAE-Cellulose Column Chromatography The dialyzed enzyme sample was applied to a column of DEAE-cellulose (Whatman DE52, 10 × 1 cm) which had been pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.5). After the column was washed with 15 ml of 10 mM Tris-HCl buffer (pH 8.5), it was eluted with a linear gradient obtained by introducing 40 ml of 25 mM Tris-HCl buffer (pH 8.5) into 40 ml of 25 mM Tris-HCl buffer (pH 8.5) containing 0.1 M NaCl at a flow rate of 7 ml/h. The enzymically active fractions were concentrated to 3 ml using a collodion membrane bag.

Step 5: Second Ultrogel AcA 34 Gel Filtration. The concentrated enzyme solution was loaded onto the same column of Ultrogel AcA 34 under the same experimental conditions, and the fractions containing high catalase activity were collected and concentrated with a collodion membrane bag. The specific activity of the concentrated enzyme preparation after Ultrogel AcA 34 was 121 kunit/mg protein.

Preparation of Glyoxysomal and Leaf Peroxisomal Fraction. The dialyzed supernatant fractions of glyoxysomes (see above) was used for immunochemical analysis as the glyoxysomal fraction. The leaf peroxisomal fraction was prepared from green pumpkin cotyledons following the same procedures for the glyoxysomal fraction. A modification employed was that the concentration of sucrose in the homogenizing buffer was raised to 15% (w/v).

Enzyme Assays. Catalase was assayed following the method of Lück (18). The reaction mixture contained 50 mM KPB (pH 7.0) and about 1.25×10^{-2} M H_2O_2. The *A* of the reaction mixture was about 0.5 at 240 nm. The first order reaction rate of H_2O_2 decomposition was monitored at 240 nm, and the unit of catalase activity was expressed either as μmol H_2O_2 broken down per min or kunits (mmol H_2O_2 broken down per min).

Other enzymes and chemical assays were those described in the literature as follows: alcohol dehydrogenase (27), Chl (24), fumarase (24), isocitrate lyase (3), protein amounts (17), ribulose-1,5-bisphosphate carboxylase (24), and xanthine oxidase (26).

Gel Electrophoresis. Three per cent polyacrylamide–0.5% agarose gel electrophoresis was carried out as described by Schiefer *et al.* (28). SDS-polyacrylamide gel electrophoresis was conducted according to the method described by Laemmli (15).

Preparation of Antiglyoxysomal Catalase IgG. Methods of preparing specific antiserum against glyoxysomal catalase were essentially the same as reported previously (23). The antigen solution containing 200 to 300 μg of the purified catalase was mixed with an equal volume of Freund's complete adjuvant. The mixture was injected into a rabbit once a week for 4 consecutive weeks. Antiserum against glyoxysomal catalase was purified to the IgG fraction by 33 to 40% $(NH_4)_2SO_4$ fractionation and subsequent DEAE-cellulose column chromatography (22).

Immunochemical Analysis. *Ouchterlony Double Immunodiffusion.* The specificity of the IgG was tested by means of the Ouchterlony double immunodiffusion, and the cross-reactivities were tested by various antigen-antibody combinations. The precipitin line was observed after 12 to 24-h incubation of the plate at 25°C.

Immunotitration. Glyoxysomal and leaf peroxisomal fractions prepared by sucrose density gradient centrifugation were dialyzed against 50 mM KPB (pH 8.0) containing 1 mM EDTA to rupture microbody membranes as well as to remove the low mol wt substances. After centrifugation (100,000*g*, 1 h) of the whole dialysate, the supernatant was used for the starting material. Varying amounts of either glyoxysomal or leaf peroxisomal fraction were added to 10 μl of anti-catalase IgG (diluted with 60 volumes of PBS). Volumes were adjusted so as to make the activities (units) of all preparations equal by adding a suitable amount of phosphate-buffered saline (pH 7.4). The series of preparations was incubated at 4°C for 12 h. The precipitates formed were removed by centrifugation (Eppendorf Microfuge, 4 min), and the supernatant fluids were subjected to the measurement of catalase activity.

Single Radial Immunodiffusion. Single radial immunodiffusion analysis was performed following Mancini *et al.* (19). Aliquots of the anti-catalase antiserum (10, 25, or 50 μl) were added to 25 nil of 0.8% (w/v) agarose containing 150 mM NaCl and 2 mM NaN_3 and the entire mixture was poured into the mold (16 × 8 × 0.2 cm). Circular wells were punch-cut in the antiserum-agarose gel, using a needle with a 4-mm bore diameter. Each well received 10 μl of the antigenic protein solution. The diameter of the circular immunoprecipitates formed was recorded with a vernier. A standard curve was made using the purified glyoxysomal catalase.

Other Methods. Sucrose density gradient centrifugation was performed according to the method of Martin and Ames (20) in a Beckman Spinco Ultracentrifuge L2-65B using a SW 65 Ti rotor. The linear sucrose gradient (5–20%, (w/v) was made using 2.25 ml each of sucrose solution containing 50 mM KPB (pH 8.0) and 1 mM EDTA. Alcohol dehydrogenase and xanthine oxidase were used as the internal markers. Enzyme solution (0.2 ml) containing purified glyoxysomal catalase (10 μg), yeast alcohol dehydrogenase (100 μg) and cow milk xanthine oxidase (1 mg) was layered on top of the gradient. The centrifugation was carried out at 32,000 rpm for 16 h at 4°C. After centrifugation,

0.1-ml fractions were collected with an ISCO density gradient fractionator (model 640), and aliquots were assayed for catalase, alcohol dehydrogenase, and xanthine oxidase activity.

RESULTS

Enzyme Purification. The presence of multiple forms of catalase (isoenzymes) has been demonstrated in mustard (5). In order to characterize catalase in the microbodies, glyoxysomes were isolated from etiolated pumpkin cotyledons and subsequently used for the enzyme purification.

As reported previously (11), glyoxysomes (marker enzyme: catalase) were well separated from mitochondria (marker enzyme: fumarase) on linear sucrose gradients, whereas they were overlapped with plastid fractions (marker enzyme: ribulose bisphosphate carboxylase). About 50% of the total cellular catalase activity was recovered in the glyoxysomal fractions, having a peak density of 1.25 g/cm^3. As shown in Table I, 93% of catalase was found to be liberated from glyoxysomes during the overnight dialysis. Figure 1 shows the elution profile of the second Ultrogel AcA 34 column chromatography. The profile of catalase activity perfectly coincides with that of A at 280 nm. Shadowed fractions 19–22 were pooled and used as purified enzyme preparation. As shown in Figure 2, the purified enzyme preparation gave only one band in both 3% acrylamide-0.5% agarose gel (A) and SDS-polyacrylamide gel electrophoresis (B). The results indicate that the pumpkin glyoxysomal catalase consists of only one type of subunit. Table I summarizes the purification of glyoxysomal catalase starting from homogenates prepared from etiolated pumpkin cotyledons. At the final stage, the enzyme was purified 288-fold, and the specific activity was 121 kunit/mg protein under the standard assay condition.

Molecular Weight. Molecular weight of the monomeric subunit of the enzyme protein was determined to be 55,000 D from the SDS-polyacrylamide gel electrophoresis (data not shown), and that of the native holoenzyme analyzed by sucrose density gradient centrifugation after Martin and Ames (20), gave a value of 230,000 D from its sedimentation value of 11.6S (Fig. 3). Similar values were obtained from purified catalase of other sources (10, 21). From these data, it is highly likely that the glyoxysomal catalase has a tetrameric structural organization.

Absorption Spectrum. The absorption spectrum of the purified enzyme was typical of a heme protein having two major peaks at 280 and 405 nm (Soret band) and three minor peaks at 510, 530, and 625 nm, respectively (Fig. 4). The ratio of absorption peaks at 280 to 405 nm was calculated to be 1.0, lower than that reported for other plant catalase molecules (7, 9, 28).

Subcellular Localization. In order to analyze the intracellular localization of catalase in the etiolated pumpkin cotyledons, the total extracts were applied to sucrose density gradient centrifugation. As shown in Figure 5, catalase activity was detected in both glyoxysomal and supernatant fractions. It can be seen that the distribution pattern of the enzyme was very similar to that of isocitrate lyase (ICL) which is localized exclusively in glyoxysomes (3). The Ouchterlony double diffusion analysis demonstrated that the rabbit antiserum (IgG) against the purified pumpkin glyoxysomal catalase cross-reacts in an manner identical with the enzyme present in the glyoxysomal and supernatant fractions (Fig. 6a). These results strongly indicate that the catalase is exclusively localized in glyoxysomes in the etiolated pumpkin cotyledons and that enzyme detectable in the supernatant fraction is simply due to organelle disruption during the isolation step.

Immunochemical Comparison of Glyoxysomal and Leaf Peroxisomal Catalase. In the Ouchterlony double diffusion analysis (Fig. 6a), the antiserum prepared against glyoxysomal catalase gave a completely fused precipitin line with (D) purified glyoxysomal catalase, (E) glyoxysomal fraction, and (B) supernatant fraction prepared from the etiolated cotyledons, indicating that the antiserum used is monospecific to the catalase.

As shown in Figure 6b, the precipitin lines formed between antiglyoxysomal catalase IgG and either glyoxysomal and leaf peroxisomal fraction were completely fused. The immunoelectrophoresis gave the same pattern between glyoxysomal and leaf peroxisomal fractions (data not shown). These results indicate that the leaf peroxisomal catalase exhibits the same immunochemical reactivity as the glyoxysomal enzyme.

However, the data in Figure 7 clearly show that there is a difference between the glyoxysomal and leaf peroxisomal catalase in immunotitration analysis. The equivalent point of the glyoxysomal catalase against the IgG was about 2.5-fold greater than that of the leaf peroxisomal enzyme. Since immunochemical differences between the glyoxysomal and leaf peroxisomal catalase were not detected by either the Ouchterlony double immunodiffusion (Fig. 6) or the immunoelectrophoretic analysis, it is highly conceivable that the difference observed in the immunotitration experiments is due to the difference in the specific activities of catalase from glyoxysomes and leaf peroxisomes.

As a measure of analyzing the developmental formation of catalase molecule in a quantitative scale during the greening process, amounts of catalase molecules in the fractions were determined by a single radial immunodiffusion analysis and these values and catalase activity in the fractions were used for calculating the specific activity (kunit/mg catalase). Figure 8 shows the distribution pattern of catalase and its specific activities during greening analyzed by the sucrose density gradient centrifugation. The specific activities of both glyoxysomal and supernatant fractions were in a range of 80 to 90 kunit/mg catalase (Fig. 8A). These values are quite comparable to those obtained from crude homogenates and the purified enzyme of glyoxysomal origin. However, it was found that after a 4-d light period the specific activity of catalase decreased to 20 to 25 kunit/mg catalase (Fig. 8C). Since the eluate from the Sephadex G-25 column gave the same specific activity as the whole homogenate (data not shown), it is inferred that the difference in the specific activities is not due to the interferring low mol wt substances. Intermediate values of the specific activity were observed after a 2-d light period (Fig. 8B). These overall results indicate that a decrease in the specific activity occurs during the greening process

Table I. *Purification of Catalase from Etiolated Pumpkin Cotyledons*

Fraction	Volume	Enzyme Activity	Enzyme Recovery	Protein Content	Specific Activity	Purification
	ml	*kunits*	*%*	*mg*	*kunits/mg*	*-fold*
Homogenate	244	3370	100	8110	0.4	1
Glyoxysomes	82	1510	45	259	5.8	14
Soluble fraction	230	1400	42	150	9.3	22
Ultrogel AcA 34	10.2	409	12	15.3	26.7	64
DEAE-cellulose	12.6	186	5.5	2.0	93	221
Ultrogel AcA 34	9.0	120	3.6	1.0	121	288

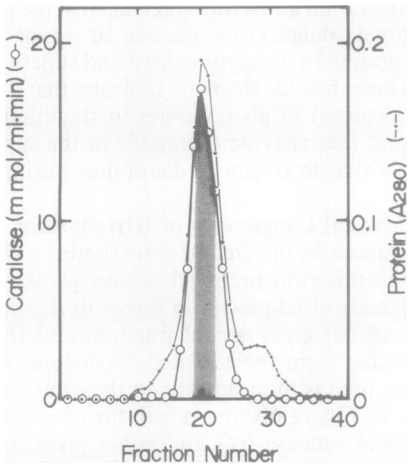

FIG. 1. Elution profile of catalase in Ultrogel AcA 34 column chromatography (Step 5). Catalase fractions isolated by DEAE-cellulose column were pooled and concentrated using a collodion membrane bag. The enzyme sample was then loaded onto a column of Ultrogel AcA 34 and eluted with 10 mM KPB (pH 8.0) containing 1 mM EDTA, and 2-ml fractions collected were used for the assay of catalase activity (O). Shadowed fractions 19–22 were collected and pooled as purified catalase preparation.

in pumpkin cotyledons. The distribution pattern of catalase from green cotyledon preparation shows that the catalase activity was recovered at a density of 1.20 and 1.25 g/cm^3, respectively (Fig. 8C). The peak at 1.20 g/cm^3 completely overlapped with that of Chl. We have applied large amounts of homogenate in order to analyze catalase content by single radial immunodiffusion technique. Therefore, catalase activities observed at a density of 1.20 g/cm^3 are likely to be derived from the leaf peroxisomes trapped by chloroplasts. It has been reported that catalase is not localized in chloroplasts (25). The trapping of mitochondria by chloroplasts was reported in wheat leaves (8).

DISCUSSION

Molecular Properties of Pumpkin Glyoxysomal Catalase. Some properties of catalase molecules isolated from various plant sources are summarized for comparison in Table II. The glyoxysomal catalase from the etiolated pumpkin cotyledons has a number of characteristics distinguishable from the enzyme of other sources. At first, its specific activity (90–120 kunit/mg protein) is higher than that of other plant enzymes except cucumber. It is possible to compare their specific activity because they were measured under similar assay conditions (Table II). The higher specific activity reported here may be explained by the different heme content of the catalase from different sources. The ratio of A at 405 and 280 nm provides a rough measure of the heme content in the enzyme (10, 21). It has been reported that the ratio of A_{280}/A_{405} for several catalases of plant origin is about 1.5 (7, 9, 28), indicating that the number of heme groups per enzyme molecule is approximately 2 (9). In contrast, the ratio with some mammalian catalase as well as the bacterial enzyme containing 4 heme groups/molecule is close to 1 (10, 21). Since the value of the pumpkin glyoxysomal catalase presently studied is approximately 1, it is conceivable that the enzyme from etiolated cotyledons has a higher heme content, similar to the animal and bacterial catalases rather than the plant enzyme.

Immunochemical Comparison of Glyoxysomal and Leaf Peroxisomal Catalase. There has not been reported any detailed analytical work trying to compare the glyoxysomal and leaf peroxisomal catalase at the molecular levels. In this paper, we have shown that the leaf peroxisomal catalase detectable in the green cotyledons resembles glyoxysomal enzyme immunochemically (Fig. 6). However, results of both immunotitration (Fig. 7) and single radial immunodiffusion analyses (Fig. 8) show that the leaf peroxisomal catalase is distinguishable from the glyoxysomal enzyme. Developmental studies on the enzyme during the greening step clearly indicate that the specific activity (catalytic efficiency) of the catalase molecule gradually decreases. A similar phenomenon concerning changes in the specific activity has been reported on β-fructofuranosidase during the maturation of tomato fruits (4).

It has been known that the catalase activity dramatically decreases during the greening process (13, 29). A part of the phenomenon can be explained on the basis of the decrease in the specific enzyme activities during greening. It will be noted in this connection that the pumpkin glyoxysomal catalase has a

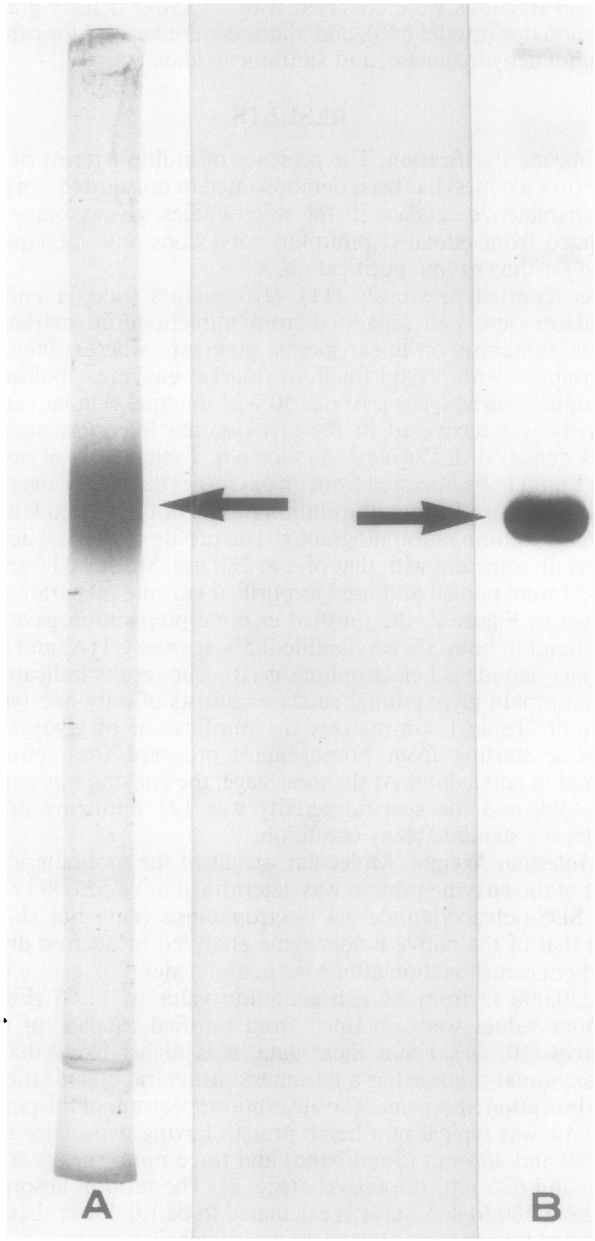

FIG. 2. Polyacrylamide gel electrophoretograms of catalase purified from etiolated pumpkin cotyledons. Purified catalase (see Fig. 1) (about 10 μg) was applied to 3% acrylamide-0.5% agarose gel (A) and SDS-polyacrylamide gel electrophoresis (B) followed. The gel was stained with Coomassie brilliant blue.

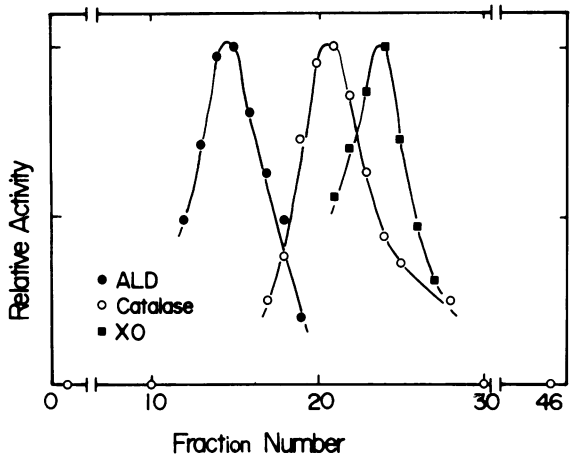

FIG. 3. Sedimentation analysis of catalase in a sucrose density gradient centrifugation. Purified catalase from pumpkin cotyledons (see Figs. 1 and 2), alcohol dehydrogenase (ALD) ($s_{20,w}$:7.4), and xanthine oxidase (XO) ($s_{20,w}$:13.8) were layered on top of the sucrose gradient (5–20%, w/v). Rotor speed was 32,000 rpm using a Beckman Spinco SW 65 Ti rotor.

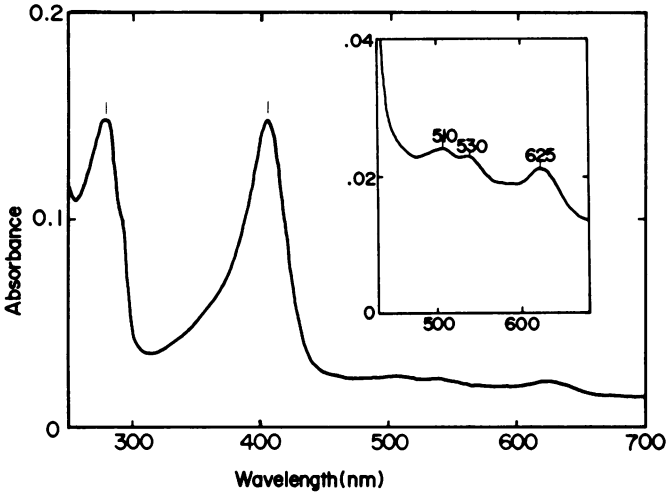

FIG. 4. Absorption spectrum of pumpkin glyoxysomal catalase. Purified catalase solution (0.11 mg/ml) was used for measurement.

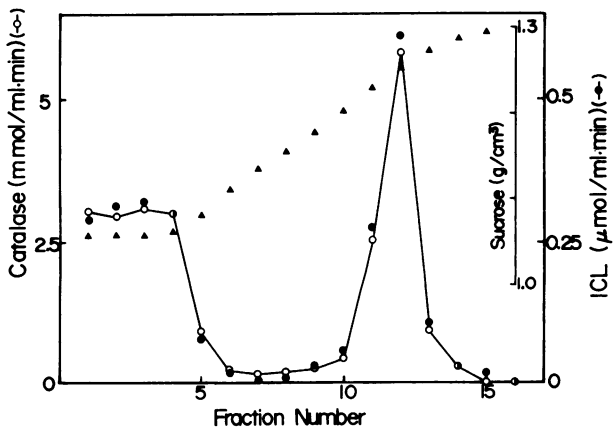

FIG. 5. Separation pattern of catalase and isocitrate lyase during sucrose density gradient centrifugation. (○), Catalase; (●), isocitrate lyase (ICL). Details of experimental conditions are described in the text.

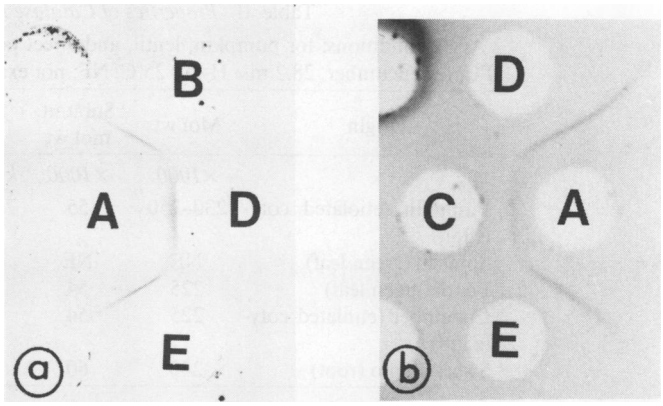

FIG. 6. Ouchterlony double immunodiffusion analysis of catalase. A, Antiglyoxysomal catalase IgG; B, supernatant fraction prepared by sucrose density gradient centrifugation from etiolated cotyledons; C, leaf peroxisomal fractions prepared by sucrose density gradient centrifugation; D, purified glyoxysomal catalase; E, glyoxysomal fraction prepared by sucrose density gradient centrifugation. Other experimental conditions are described in the text.

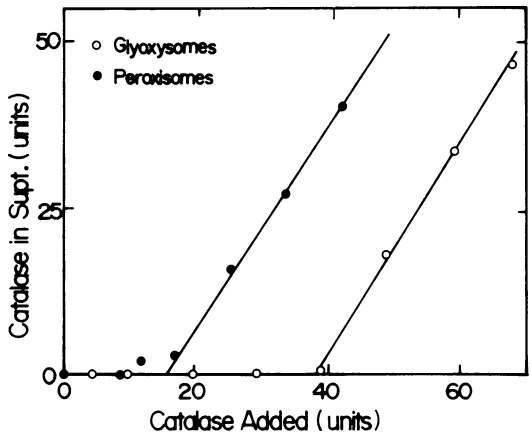

FIG. 7. Immunotitration analysis of glyoxysomal and leaf peroxisomal catalase. (○), Glyoxysomal fraction; (●), leaf peroxisomal fraction. Details of other experimental conditions are described in the text.

higher specific enzyme activity as well as the heme content in comparison with other plant enzymes. It is thus possible that the heme content in the catalase molecule changes during the step of seedling greening.

Previously Betsche and Gerhardt (2) have reported that there is no increase in the rate of biosynthesis of catalase during the greening process of sunflower cotyledons, and implicated the direct transformation of glyoxysomes to leaf peroxisomes (the one-population model) rather than the *de novo* synthesis of the latter (the two-population model). However, their postulation is principally based on the mathematical treatment of the data obtained from the density labeling of the enzyme, and importantly they had assumed that there is no change in the specific enzyme activities during greening or sunflower cotyledons. It should be emphasized that if the specific activities change in the pumpkin cotyledons as presently demonstrated, their conclusion needs to be reassessed.

Our present investigation clearly shows that the antiglyoxysomal catalase IgG cross-reacts with the leaf peroxisomal enzyme. We believe that the immunological approach for the analysis of the turnover of catalase during the greening process will provide us useful information concerning the mechanism of the microbody transition.

Table II. *Properties of Catalase Molecules of Various Plant Origins*

Assay conditions: for pumpkin, lentil, and sweet potato, 12.5 mM H_2O_2, 25°C; for spinach, 10 mM H_2O_2, 20°C; for cucumber, 28.2 mM H_2O_2, 25°C. NE: not examined.

Origin	Mol wt	Subunit mol wt	Specific Activity	A_{280}/A_{405}	Hemin/Molecule	Reference
	×1000	*×1000*	*kunits/mg*			
Pumpkin (etiolated cotyledon)	230–250	55	90–120	1.0	NE	
Spinach (green leaf)	NE	NE	28	1.5	2	9
Lentil (green leaf)	225	54	30	1.5	NE	28
Cucumber (etiolated cotyledon)	225	54	68	NE	NE	16
Sweet potato (root)	240	60	28	1.5	NE	7

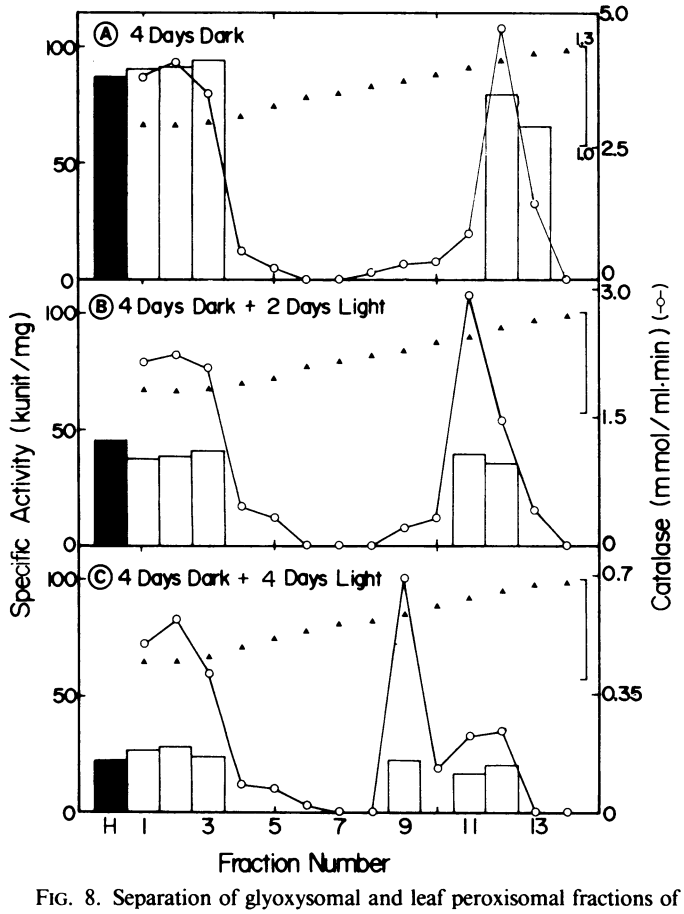

FIG. 8. Separation of glyoxysomal and leaf peroxisomal fractions of etiolated and green pumpkin cotyledons by sucrose density gradient centrifugation and determination of specific activities of catalase. A, Cotyledons of dark-grown (4 d) seedling; B, cotyledons of dark-grown (4 d) and then illuminated for 2 d; C, same as B but illuminated for 4 d. Twenty pairs of cotyledons were homogenized by chopping with a razor blade in 5 to 8 ml of homogenizing medium described in the text. Three ml of chopped homogenates were applied to the sucrose gradient (2 ml of 20% (w/w), 12 ml of linear 30 to 60% (w/w) containing 1 mM EDTA) and centrifugation was performed at 21,000 rpm for 3 h using a Beckman Spinco SW 27-1 rotor with Beckman L2-65B Ultracentrifuge. At the end of centrifugation 1.2-ml fractions were collected with an ISCO density gradient fractionator (model 640), and subjected to the single radial immunodiffusion analysis and the assay of catalase activities (O). Bars represent the specific activities of catalase (kunit/mg catalase protein) in each fraction. H, Crude homogenate.

Acknowledgment—The authors with to thank Dr. T. Akazawa for his support and stimulating discussions.

LITERATURE CITED

1. BEEVERS H 1979 Microbodies in higher plants. Annu Rev Plant Phyiol 30: 159–193
2. BETSCHE T, B GERHARDT 1978 Apparent catalase synthesis in sunflower cotyledons during the change in microbody function. A mathematical approach for the quantitative evaluation of density-labeling data. Plant Physiol 62: 590–597
3. COOPER TG, H BEEVERS 1969 Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. J Biol Chem 244: 3507–3513
4. DAUSSANT J, A SKAKOUN 1981 Immunochemical approaches to studies of isoenzyme regulation in higher plants. *In* MC Ratazzi *et al*, eds, Isoenzymes: Current Topics in Biological and Medical Research, Vol 5. Alan R. Liss, New York, pp 175–218
5. DRUMM H, P SCHOPFER 1974 Effect of phytochrome on development of catalase activity and isoenzyme pattern in mustard (*Sinapis alba L.*) seedlings. Planta 120: 13–30
6. EMDEN L 1947 Über Kürbis-Katalase. Helv Chem Acta 30: 15–19
7. ESAKA M, T ASAHI 1982 Purification and properties of catalase from sweet potato root microbodies. Plant Cell Physiol 23: 315–322
8. FEIERABEND J, H BEEVERS 1972 Developmental studies of microbodies in wheat leaves. II. Ontogeny of particulate enzyme associations. Plant Physiol 49: 33–39
9. GALSTON AW, RT BONNICHSEN, DI ARNON 1951 The preparation of highly purified spinach leaf catalase. Acta Chem Scand 5: 781–790
10. HERBERT D, J PINSENT 1948 Crystalline human erythrocyte catalase. Biochem J 43: 193–202
11. HUANG AHC, H BEEVERS 1971 Isolation of microbodies from plant tissues. Plant Physiol 48: 637–641
12. HUANG AHC, H BEEVERS 1973 Localization of enzymes within microbodies. J Cell Biol 58: 379–389
13. KAGAWA T, H BEEVERS 1975 The development of microbodies (glyoxysomes and leaf peroxisomes) in cotyledons of germinating watermelon seedlings. Plant Physiol 55: 258–264
14. KAGAWA T, JM LORD, H BEEVERS 1975 Lecithin synthesis during microbody biogenesis in watermelon cotyledons. Arch Biochem Biophys 167: 45–53
15. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
16. LAMB JE, H RIEZMAN, WM BECKER 1978 Regulation of glyoxysomal enzymes during germination of cucumber. 2. Isolation and immunological detection of isocitrate lyase and catalase. Plant Physiol 62: 754–760
17. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
18. LÜCK H 1965 Catalase. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis. Academic Press, New York, pp 885–894
19. MANCINI G, AO CARBONARA, JF HEREMANS 1965 Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2: 235–254
20. MARTIN RG, BN AMES 1961 A method of determining the sedimentation behavior of enzymes: application to protein mixtures. J Biol Chem 236: 1372–1379
21. NICHOLIS P, GR SCHONBAUM 1963 Catalases. In PD Boyer, ed, The Enzymes, Ed 2, Vol 8. Academic Press, New York, pp 147–225
22. NISHIMURA M, T AKAZAWA 1974 Studies on spinach leaf ribulose bisphosphate carboxylase. Carboxylase and oxygenase reaction examined by immunochemical methods. Biochemistry 13: 2277–2281
23. NISHIMURA M, Y AKHMEDOV, T AKAZAWA 1983 Molecular structure and subcellular localization of spinach leaf glycolate oxidase. Photosynth Res: 4: 99–109
24. NISHIMURA M, P BHUSAWANG, K STRZALKA, T AKAZAWA 1982 Developmental formation of glutamine synthetase in greening pumpkin cotyledons and its subcellular localization. Plant Physiol 70: 353–356
25. NISHIMURA M, D GRAHAM, T AKAZAWA 1976 Isolation of intact chloroplasts and other organelles from spinach leaf protoplasts. Plant Physiol 58: 309–

314
26. PRICE VE, MC OTEY, P PLESNER 1955 Preparation of nucleoside phosphorylase from calf spleen. Methods Enzymol 2: 448–453
27. RACKER E 1955 Alcohol dehydrogenase from baker's yeast. Methods Enzymol 1: 500–503
28. SCHIEFER S, W TEIFEL, H KINDL 1976 Plant microbody proteins. I. Purification and characterization of catalase from leaves of *Lens culinaris*. Z Physiol Chem 357: 163–175
29. TRELEASE RN, WM BECKER, PJ GRUBER, EH NEWCOMB Microbodies (glyoxysomes and peroxisomes) in cucumber cotyledons. Correlative biochemical and ultrastructural study in light- and dark- grown seedlings. Plant Physiol 48: 461–475