

Geranoyl-CoA Carboxylase: A Novel Biotin-Containing Enzyme in Plants

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Geranoyl-CoA carboxylase (EC 6.4.1.4) is a biotin-containing enzyme previously described in two genera of bacteria. Here we report the presence of geranoyl-CoA carboxylase in kingdom Plantae. Geranoyl-CoA carboxylase was purified 180-fold from maize leaves. The enzyme has a biotin-containing subunit of 122 kDa. The pH optimum for activity is 8.3. The apparent K_m values for the substrates geranoyl-CoA, bicarbonate, and ATP are $64 \pm 5 \mu\text{M}$, $0.58 \pm 0.04 \text{ mM}$, and $8.4 \pm 0.4 \mu\text{M}$, respectively. Subcellular fractionations indicate that geranoyl-CoA carboxylase is located in plastids. Geranoyl-CoA carboxylase activity is ubiquitous in organs of monocots and dicots and varies with development. We postulate that geranoyl-CoA carboxylase plays an important role in isoprenoid catabolism in plants, in a pathway analogous to that shown in *Pseudomonas* sp. In plants, this catabolic pathway would require the interaction of at least three subcellular compartments (plastids, microbodies, and mitochondria) and two biotin-containing enzymes, geranoyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase.

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Key Words: geranoyl-CoA carboxylase; *Zea mays*; *Daucus carota*; biotin; plastids; chloroplasts; isoprenoids.

Geranoyl-CoA carboxylase (GCCase)⁵ is a biotin-containing enzyme that catalyzes the ATP-dependent carboxylation of the methyl group that is proximal to the carboxyl end of the monoterpene, geranoyl-CoA, to form γ -carboxygeranoyl-CoA (Fig. 1) (1–4). This enzyme has been reported only in a few prokaryotic microorganisms: *Pseudomonas citronellolis* [where GCCase was originally discovered by Seubert (3)], *P. aeruginosa*, *P. mendocina*, and *Acinetobacter* (5). In these bacteria, GCCase is required for a pathway that degrades isoprenoids such as citronellol, nerol, and geraniol (5). GCCase activity is induced when these bacteria are supplied with isoprenoids as the sole carbon source (5).

GCCase has been purified from *P. citronellolis* (2, 6, 7). The purified enzyme is composed of two nonidentical subunits, a biotin-containing subunit of 75 kDa and a nonbiotinylated subunit of 63 kDa (7). The molecular weight of the holoenzyme is estimated at 520,000–580,000; hence, the enzyme appears to have an $\alpha_4\beta_4$ quaternary structure (4, 7).

In this paper, we report the discovery of GCCase in plants. Biochemical and physiological characterizations reveal that the plant GCCase has properties that are distinct from those of the *Pseudomonas* enzyme. The discovery of GCCase in plants indicates that isoprenoids may be degraded in these organisms via the carboxylation of geranoyl-CoA.

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⁵ Abbreviations used: ACCase, acetyl-CoA carboxylase; CoA, coenzyme A; DAP, days after planting; DTT, dithiothreitol; GCCase, geranoyl-CoA carboxylase; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; MCCase, 3-methylcrotonyl-CoA carboxylase; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; Rubisco, ribulose-1,5-bisphosphate carboxylase.

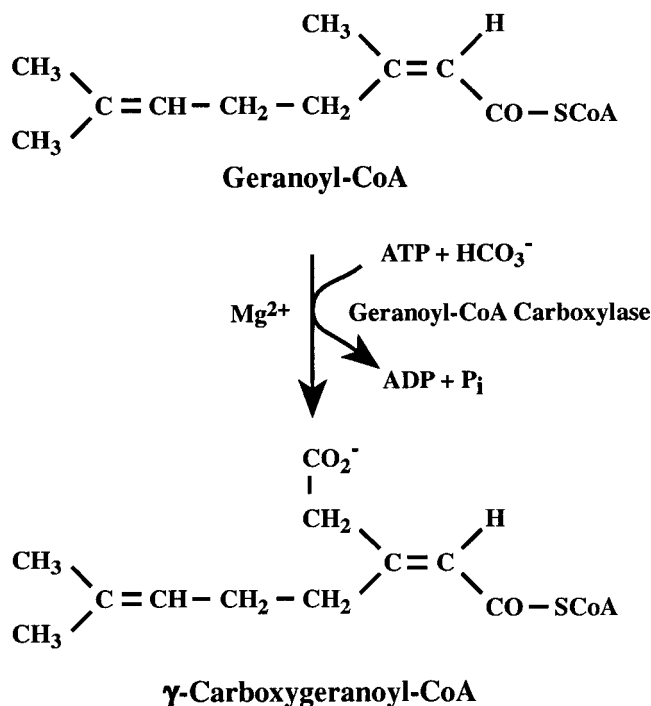


FIG. 1. Reaction catalyzed by GCCase.

MATERIALS AND METHODS

Reagents

$\text{NaH}^{14}\text{CO}_3$ (53.1 Ci/mol) was purchased from Amersham. Streptavidin and protein A were radioactively labeled with carrier-free Na^{125}I to specific radioactivities of about 2×10^7 dpm/ μg (8).

Plant Materials

GCCase was purified from 10- to 14-day-old maize (*Zea mays L.*) seedlings grown in a greenhouse as previously described (9). Plastids were isolated from maize seedlings (inbred G50; Pioneer Hi-Bred International Inc., Johnston, IA) grown in a dark growth chamber at 27°C for 3 days (10). Chloroplasts were isolated from 5-day-old maize seedlings grown initially in darkness for 3 days and subsequently grown in a greenhouse for another 2 days (10).

Soybean seeds [*Glycine max* (L.) Merr. cv Corsoy 79], kindly provided by Dr. I. C. Anderson, Department of Agronomy, Iowa State University, were planted in 16-cm-high pots containing sterilized soil (a 1:1:1 mixture of soil:peat:perlite). Soybean seedlings were grown in a greenhouse at 22 to 24°C, with a maximum daily irradiance of no less than 1200 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, and watered daily. Because of potential variations in enzyme activity during the diurnal cycle, plant samples used for developmental studies were harvested at about 3:30 PM. Carrot (*Daucus carota L.*) cells and carrot embryos were cultured as described previously (11, 12). Immediately upon harvest, tissues were frozen in liquid nitrogen.

Synthesis of Geranyl-CoA

All the reactions were carried out at room temperature. Geranoic acid was prepared by oxidation of citral with Ag_2O as described by Shamma and Rodriguez (13). Geranyl-CoA was synthesized by the mixed anhydride method of Stadman (14), with slight variations.

Briefly, 770 μmol of geranoic acid was dissolved in 5.1 ml of tetrahydrofuran and neutralized with an equimolar amount of triethylamine. Ethylchloroformate (770 μmol) was added and the mixture was allowed to react for 30 min. The mixture was then filtered through glasswool and the anhydride was recovered in the filtrate. The mixed anhydride compound was added dropwise, over a period of 15 min, to coenzyme A (29 μmol) dissolved in 12 ml of H_2O :tetrahydrofuran, 3:2 (v/v), which had previously been neutralized to pH 8.0 with solid NaHCO_3 . The mixture was stirred, and to avoid phase separation a few milliliters of H_2O was added dropwise. After 20–25 min, the pH of the mixture was adjusted to 3.0 with 2 N HCl. The unreacted mixed anhydride was removed by three extractions with equal volumes of ethyl ether. Finally, the remaining tetrahydrofuran was eliminated by evaporation under vacuum and geranyl-CoA was recovered by lyophilization. Routinely, 12–15% of the CoA was recovered as geranyl-CoA. The lyophilized geranyl-CoA was dissolved in 1 mM Mes and its concentration was determined by the hydroxamate method of Lipmann and Tuttle (15). The purity of the synthesized geranyl-CoA was established by reverse-phase HPLC using a Supelcosil LC-18 column (25 cm \times 4.6 mm i.d., 5- μm particle size), with a solvent gradient system previously described (16). This chromatography procedure was also used to characterize the GC-Case reaction product.

Preparation of Cell-Free Extracts

Cell-free extracts were prepared essentially as described by Wurtele and Nikolau (17). Frozen tissues were homogenized to a fine powder with a mortar and pestle under evaporating liquid nitrogen. After evaporation of the nitrogen, the powder was resuspended in 3 vol of 100 mM Hepes-KOH, pH 7.0, 1 mM EDTA, 2 mM DTT, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM E64, and 100 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride. The mixture was centrifuged at 10,000g for 20 min at 4°C, and the supernatant was collected. Two hundred microliters of this protein extract was passed through a 1-ml Sephadex G-25 column preequilibrated in 10 mM Hepes-KOH, pH 7.0, 1 mM EDTA, 2 mM DTT, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM E64 (18). Forty microliters of the size-excluded eluate, containing 20–80 μg protein, was used for each enzyme assay.

Large-scale extractions (200–250 g fresh wt) were achieved with 3 vol of Hepes buffer solution (as above) using a Waring blender (9).

Purification of GCCase

The purifications were performed at 4°C, from 200–250 g fresh wt of maize leaves.

Step 1: PEG precipitation. GCCase was collected from the crude extract by precipitation with PEG 8000 at a final concentration of 18 g/100 ml. The mixture was slowly stirred until the PEG was completely dissolved. The suspension was centrifuged at 12,200g for 25 min. The pellet was collected and immediately dissolved in 50–75 ml of buffer A, which consisted of 10 mM Hepes-KOH (pH 7.0), 10 mM 2-mercaptoethanol, 1 mM EDTA, and 20% glycerol (v/v) (PEG fraction).

Step 2: Hydrophobic interaction chromatography on propyl-agarose. The PEG fraction was applied to a propyl-agarose column (1.6 \times 20 cm), previously equilibrated with buffer A. The column was then washed with 200 ml of buffer A. Elution of GCCase was carried out with an 800-ml linear gradient of 0 to 0.5 M KCl in buffer A. The fractions containing GCCase were pooled and frozen in liquid nitrogen (hydrophobic fraction).

Step 3: Affinity chromatography on Cibacron-Blue 3GA agarose. The hydrophobic fraction was applied to a column of Cibacron-Blue 3GA agarose (1.6 \times 10 cm) previously equilibrated with buffer A. The unbound proteins were removed by washing the column with 200 ml

of buffer A containing 0.075 M KCl. GCCase was then eluted with an 800-ml linear gradient of 0.075 to 0.75 M KCl in buffer A. Fractions containing GCCase were pooled (Cibacron-Blue fraction).

Step 4: Ion-exchange chromatography on Q-Sepharose. The Cibacron-Blue fraction was dialyzed against 8 liters of buffer A for 4–5 h. The dialyzed Cibacron-Blue fraction was then applied to a Q-Sepharose column (2.6 × 18 cm) previously equilibrated with buffer A. The column was washed with 100 ml of buffer A and GCCase was eluted with an 800-ml linear gradient of 0 to 0.5 M KCl in buffer A. Fractions containing GCCase activity were frozen in liquid nitrogen and stored at –80°C.

Preparation of Subcellular Organelles

Subcellular organelles were isolated from maize seedlings as described by Prasad *et al.* (10). Briefly, 15 g fresh wt of mesocotyls from 3-day-old dark-grown maize seedlings was homogenized to a smooth paste in 25 ml of a grinding buffer (0.4 M sucrose, 165 mM Tricine–NaOH, pH 7.5, 10 mM KCl, 10 mM MgCl₂, 10 mM EDTA, and 1 mM DTT). The mixture was filtered through four layers of cheesecloth and centrifuged at 270g for 5 min. The resulting supernatant was centrifuged at 10,800g for 15 min. The pellet, containing plastids and mitochondria, was resuspended in 4 ml of grinding buffer and layered onto a sucrose step gradient [from bottom to top: 7 ml each of 57, 50, 44, 33, and 30% (w/w) sucrose in 165 mM Tricine–NaOH, pH 7.5, 10 mM EDTA, 10 mM KCl, 10 mM MgCl₂, and 1 mM DTT]. The gradient was centrifuged at 20,000g for 1 h at 4°C using an SW 28 rotor in a Beckman L-4 ultracentrifuge, and 1-ml fractions were collected.

Protease Digestion of Chloroplasts

Chloroplasts were isolated from the leaves of light-grown maize seedlings according to the method of Blair and Ellis (19). Intact or lysed (by repeated freeze–thaw cycles) chloroplasts were incubated with trypsin at 25°C at a ratio of 50:1 (w/w) chloroplast protein:trypsin in 0.1 M sodium phosphate buffer, pH 7.2, for 0, 15, 30, or 45 min. After incubation, the protease activity was inhibited by the addition of soybean trypsin inhibitor at a ratio of 3:1 (w/w) trypsin inhibitor:trypsin. Experiments were repeated three times with similar results; the results shown are those of a single experiment.

Assays

GCCase, acetyl-CoA carboxylase (ACCCase) (20), methylcrotonyl-CoA carboxylase (MCCCase) (9, 17), PEP carboxylase (20), and Rubisco (21) were assayed as the rate of incorporation of radioactivity from NaH¹⁴CO₃ into the acid-stable products. The pH optimum of GCCase activity was measured in the range between pH 6.3 and 10.3, using Bis–Tris–propane as the buffer. Assays of GCCase activity were performed in triplicate. Kinetic constants are the averages of two independent experiments. Cytochrome *c* oxidase (22) and fumarase (23) were assayed spectrophotometrically. Protein concentration was determined by a dye-binding method (24). Chlorophyll concentration was determined using the method of Arnon (25).

SDS-PAGE and Western Blot Analysis

SDS–PAGE was carried out according to Laemmli (26). Proteins were transferred from gels to nitrocellulose filters using a semidry transblot apparatus according to the instructions of the manufacturer (PolyBlot, American Bionetics, Inc.) and biotin-containing polypeptides were detected with ¹²⁵I-labeled streptavidin (8).

TABLE I
Substrate Requirements for GCCase Activity in Extracts from Maize Seedlings

Omission or addition	Acid-stable radioactivity (dpm)
None	14,030
– geranyl-CoA	570
– ATP	480
– MgCl ₂	1,400
+ avidin	2,230
+ (avidin + biotin)	14,510

Note. GCCase activity was determined as described under Materials and Methods. Each reaction mixture was identical, with the exception of the indicated omission or addition, and contained an aliquot of a maize seedling extract constituting 60 μg of protein. Following a 10-min incubation, the amount of acid-stable radioactivity was determined.

RESULTS

GCCase Is a Novel Biotin-Containing Enzyme in Plants

Initial experiments were undertaken to ascertain if GCCase activity could be detected in extracts of maize leaves. As shown in Table I, maize leaf extracts can catalyze the conversion of radioactivity from NaH¹⁴CO₃ into an acid-stable product, consistent with the presence of GCCase. HPLC analyses (16) showed the presence of a single radioactive product. This compound was alkali labile as expected for an acyl-CoA-derivative. In addition, this compound coeluted with the authentic GCCase product, γ-carboxygeranyl-CoA, prepared by carboxylation of geranyl-CoA by extracts of *P. citronellolis* (4). This activity has an absolute requirement for ATP, Mg²⁺, and geranyl-CoA. In addition, this carboxylation reaction is biotin dependent, since it is completely inhibited by avidin; the inhibition by avidin is prevented by the incubation of avidin with biotin prior to its addition to the assay mixture (Table I). GCCase activity was also detected in extracts from leaves of *Nicotiana tabacum*, *Daucus carota*, and *Glycine max* (data not shown). These results demonstrate that the biotin-containing enzyme GCCase is present in the plant kingdom.

Purification of GCCase

A procedure for the purification of GCCase from extracts of maize leaves was developed (Figs. 2 and 3, and Table II). Nearly 100% of the GCCase activity found in the crude extract was recovered in the pellet precipitated by 18% PEG. Fractionation of the PEG fraction by hydrophobic interaction chromatography on propyl-agarose resulted in a GCCase preparation, which also contained MCCase activity (Fig. 2A). MCCase is a bi-

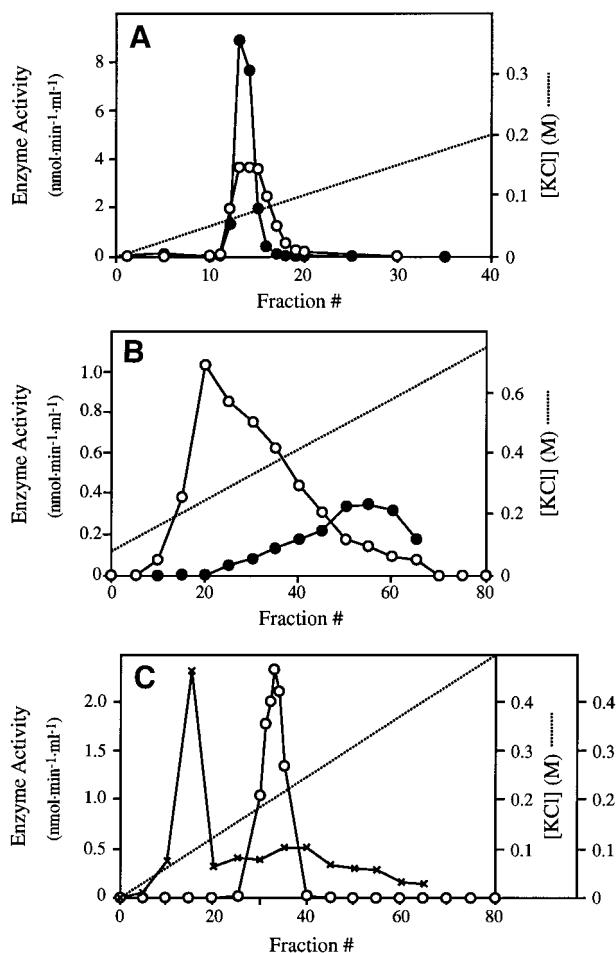


FIG. 2. Chromatographic fractionation of GCCase and MCCase activities from maize leaf extracts. (A) Fractionation of MCCase and GCCase activities by hydrophobic chromatography. (B) Fractionation of MCCase and GCCase activities by affinity chromatography on Cibacron-Blue agarose. (C) Elution of GCCase activity from a Q-Sepharose. GCCase (○) and MCCase (●) activities.

otin-containing enzyme that catalyzes the carboxylation of methylcrotonyl-CoA to form methylglutaconyl-CoA and may be involved in leucine catabolism (9, 17, 27). The similar elution profiles of GCCase and MCCase activities, as well as the similarities between the chemical structure of the substrates of these two enzymes, raised the possibility that these two activities may be catalyzed by the same enzyme. This possibility was discounted following elution of GCCase and MCCase activities from the Cibacron-Blue affinity matrix (Fig. 2B). GCCase and MCCase activities were clearly resolved, indicating that they are distinct enzymes.

The Cibacron-Blue fractions that contained GCCase but lacked MCCase were pooled, and the pooled fractions were subjected to ion-exchange chromatography on Q-Sepharose (Fig. 2C). GCCase eluted from this column at a salt concentration of 0.2 M KCl, with a

peak specific activity of 138 nmol/min mg protein. Thus, GCCase was purified approximately 180-fold, with a 4% recovery.

The recovery of protein in the fractions containing GCCase was too low for the subunit(s) of this enzyme to be detected by Coomassie or silver staining following SDS-PAGE. However, SDS-PAGE followed by Western blot analysis with 125 I-labeled streptavidin revealed a single biotin-containing polypeptide, with a molecular weight of 122,000, in the Q-Sepharose fraction with peak GCCase activity. Indeed, as GCCase is purified this 122-kDa biotin-containing polypeptide is enriched (Fig. 3). Thus, we conclude that maize GCCase has a 122-kDa biotin-containing subunit.

Kinetic Characterization

Increasing concentration of each substrate results in a hyperbolic increase in GCCase activity, indicating that maize leaf GCCase follows classical Michaelis-

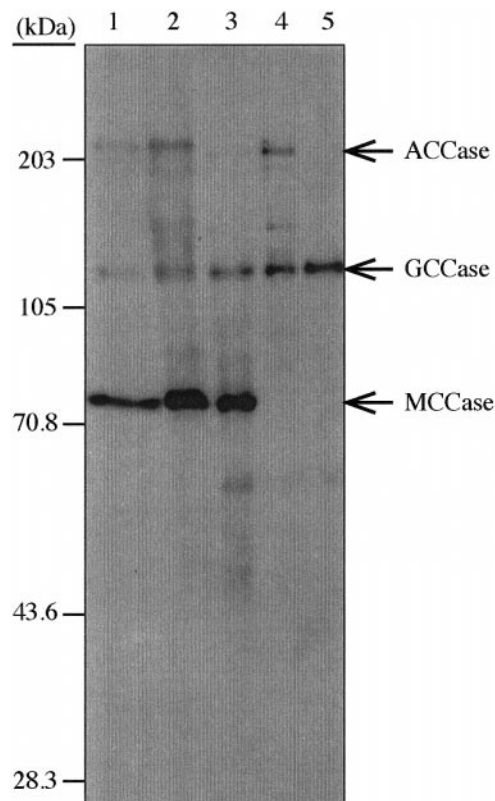


FIG. 3. The biotin-containing subunit of GCCase. Individual fractions from the GCCase purification protocol (described under Materials and Methods) were subjected to SDS-PAGE and Western analyses with 125 I-labeled streptavidin. Lane 1, extract; lane 2, PEG fraction; lane 3, hydrophobic fraction; lane 4, Cibacron-Blue fraction; lane 5, Q-Sepharose fraction.

TABLE II
Purification of GCCase from Maize Leaves

Fraction	Total protein (mg)	Total activity (nmol min ⁻¹)	Recovery (%)	Specific activity (nmol min ⁻¹ mg ⁻¹)	Purification (fold)
Extract	720	540	100	0.8	1.0
PEG	530	533	99	1.0	1.3
Hydrophobic	49	463	86	9.4	12
Cibacron blue	8	196	36	25.3	34
Q-Separeose	0.15	21	4	138.2	184

Note. Purification procedures are described under Materials and Methods.

Menten kinetics (Fig. 4). The K_m and V_{max} for each substrate were determined from Lineweaver-Burk analysis of the data. Maize GCCase is active over a broad pH range, with maximum activity occurring at pH 8.3 (Fig. 4D).

Subcellular Localization of GCCase

Plastids and mitochondria were purified from homogenates of mesocotyls of dark grown maize seedlings by sucrose density gradient centrifugation (10). Assays of fractions from the gradient indicate that GCCase

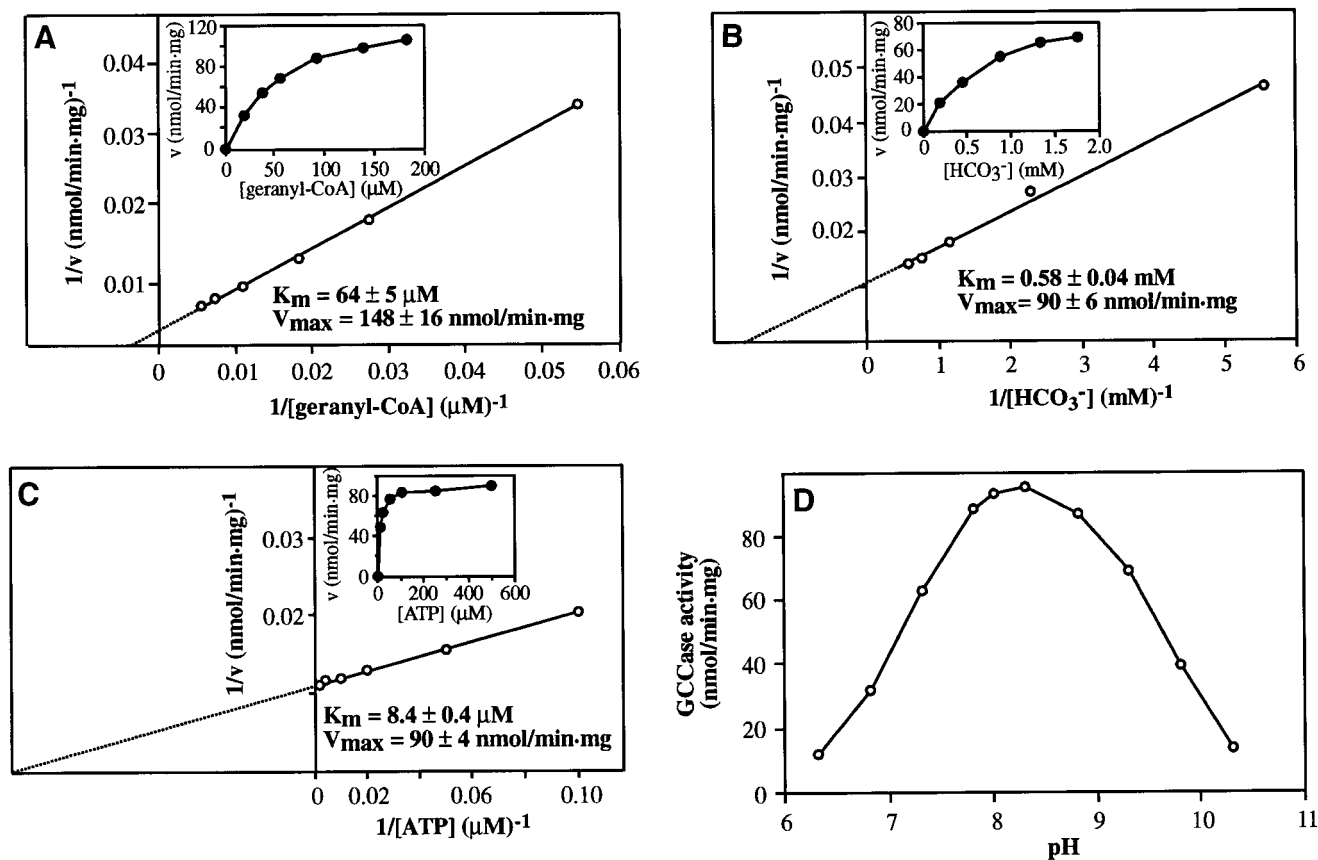


FIG. 4. Kinetic characterizations of maize GCCase. (A) Lineweaver-Burk analysis of GCCase dependence on geranyl-CoA concentration; inset shows the response of the enzyme to increasing substrate concentration. (B) Lineweaver-Burk analysis of GCCase dependence on bicarbonate concentration; inset shows the response of the enzyme to increasing substrate concentration. (C) Lineweaver-Burk analysis of GCCase dependence on ATP concentration; inset shows the response of the enzyme to increasing substrate concentration. (D) Response of GCCase activity to change in pH. Unless otherwise indicated, GCCase activity was determined in a buffer consisting of 50 mM Tricine-KOH (pH 8.3), 5 mM NaHCO₃, 2.5 mM MgCl₂, 0.5 mM ATP, 1 mM DTT, and 0.2 mM geranyl-CoA.

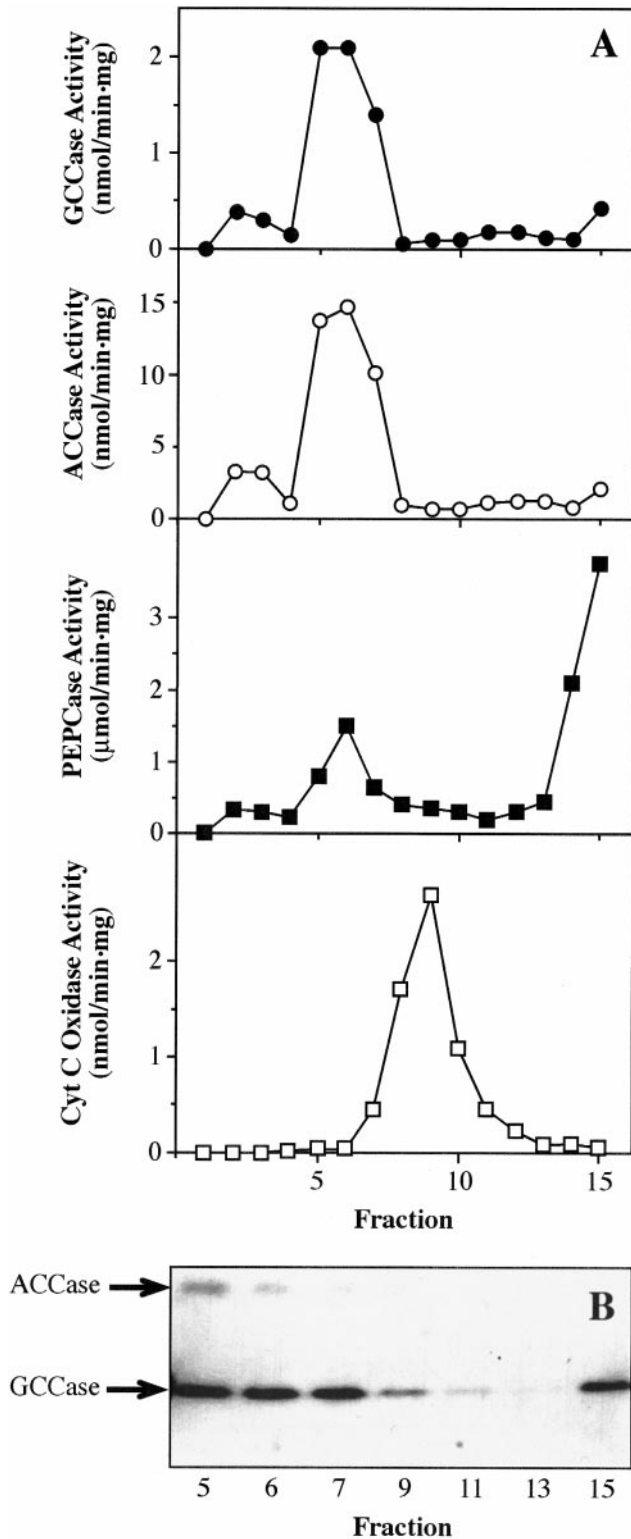


FIG. 5. Localization of GCCase in chloroplasts of maize leaves. A crude intact plastid fraction isolated from maize leaves was subjected to sucrose gradient density centrifugation. (A) Fractions from this gradient were assayed for GCCase, ACCase (enzyme marker of chloroplasts), PEP carboxylase (enzyme marker of cytosol), and

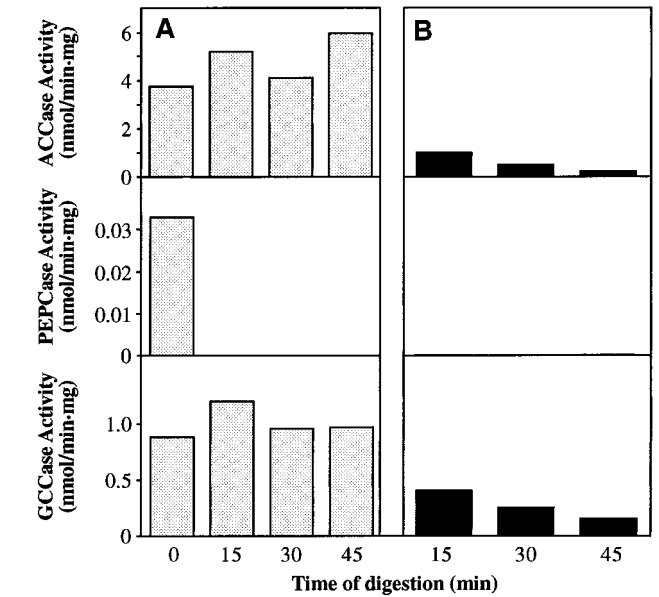


FIG. 6. Localization of GCCase within plastids of maize leaves. (A) Isolated intact chloroplasts were incubated with trypsin in an isotonic medium. (B) Isolated intact chloroplasts were lysed by repeated freeze-thaw cycles and incubated with trypsin in an isotonic medium. Residual GCCase, ACCase, and PEP carboxylase activities were determined following the indicated digestion times.

activity is associated with the plastids (Fig. 5A). Western analysis using 125 I-labeled streptavidin as a probe indicates that the 122-kDa biotinylated subunit of GCCase cofractionates with the plastids (Fig. 5B). Additional organelle fractionations using protoplasts from carrot cells were consistent with the results from maize; at least 84% of GCCase activity was recovered in the carrot plastids (28).

To confirm that GCCase is located within the plastids, as opposed to adhering to the envelope during plastid purification, intact purified chloroplasts from light-grown maize seedlings (10) were incubated with trypsin for various lengths of time. In these experiments, ACCase activity was used as a marker for mesophyll cell chloroplasts (20), and PEP carboxylase was used as a cytosolic marker. A low level of PEP carboxylase activity was associated with the plastids isolated from maize seedlings (Figs. 5 and 6), presumably adhering to the outer chloroplast membrane as a cytosolic contaminant. Following incubation with trypsin, PEP carboxylase activity was completely eliminated from the purified intact chloroplast preparations, as would

cytochrome *c* oxidase (enzyme marker of mitochondria). (B) Distribution of biotin-containing proteins among the fractions from the identical gradient was visualized by SDS-PAGE and Western analyses with 125 I-labeled streptavidin.

TABLE III
GCCase Distribution among Plant Tissues

Plant tissue	GCCase activity ^a nmol (min mg protein) ⁻¹
Maize (7-day-old seedling) ^a	
Roots	1.0 ± 0.3
First leaf blade	0.85 ± 0.08
First leaf base	0.61 ± 0.06
Second leaf blade	0.8 ± 0.3
Second leaf base	1.0 ± 0.3
Coleoptile	0.30 ± 0.02
Soybean (4-day-old seedling)	
Root tip	0.5 ± 0.8
Mature root	0.5 ± 0.8
Hypocotyl	0.2 ± 0.3
Cotyledon	0.88 ± 0.09
Soybean (15-day-old seedling)	
Root	0.60 ± 0.08
Hypocotyl	0.35 ± 0.02
Cotyledon	0.10 ± 0.02
Stem	0.18 ± 0.02
Unifoliolate leaf	0.031 ± 0.006
First trifoliolate leaf	0.24 ± 0.04
Shoot apex	0.34 ± 0.03
Immature seed	0.16 ± 0.03

Note. GCCase activity was determined in extracts from the indicated plant tissues.

^a Data are means ± SD of three determinations.

be expected for a cytosolic enzyme (Fig. 6). However, both GCCase and ACCase activities were unaffected. In contrast, when chloroplasts were lysed by repeated cycles of freezing and thawing prior to incubation with trypsin, both GCCase and ACCase activities were susceptible to digestion. These results establish that GCCase is localized within plastids. These data do not exclude the possibility that additional GCCase isozymes may be present in other subcellular compartments.

Distribution of GCCase Activity among Plant Organs

To obtain insights into the role of GCCase in plant metabolism, the relative distribution of GCCase activity was determined among organs at different developmental stages. These studies were carried out with representative monocot (maize) and dicot (soybean and carrot) species.

In organs of 7-day-old maize seedlings, GCCase specific activity was present at similar levels in all organs examined. GCCase specific activity was considerably lower in extracts of soybean than maize (Table III). However, there was considerable variation in the specific activity of GCCase in different organs of the soybean plant. The highest specific activities were found in extracts from non-photosynthetic organs, namely

roots, hypocotyls, and the shoot apex. For example, in roots GCCase activity was about 20-fold higher than in unifoliolate leaves and 6-fold higher than in cotyledons.

During seedling development, soybean cotyledons progress through physiologically distinct stages (germination, expansion and greening, and senescence) associated with major redistributions of carbon via different metabolic pathways (29). At the end of germination, when the cotyledons are still underneath the soil (3 DAP), GCCase activity was relatively low (Fig. 7). As cotyledons begin to expand and acquire chlorophyll, GCCase activity increased almost threefold, reaching maximal activity at 12 DAP, coincident with the maximal size and chlorophyll content of the cotyledons. GCCase activity subsequently decreased between 12 and 18 DAP, while Rubisco activity peaked. During senescence, GCCase activity reached a second peak at about 30 DAP, just before cotyledon abscission.

To establish whether GCCase activity is associated with a particular phase of cell growth, its activity was determined in cell-free extracts from cultured carrot cells harvested at various times after subculture. GCCase activity was highest in the first 14 days after subculture, until the midlog phase of growth; subsequently, activity declined dramatically, so that by the stationary stage of growth activity was about 10% of the initial activity (Fig. 8). In addition, cells of carrot suspension cultures were induced to form embryos, which were fractionated into developmentally distinct stages (11, 12, 30). GCCase activity was highest in embryogenic cells, prior to embryogenesis, declined following initiation of embryos (threefold decline in glob-

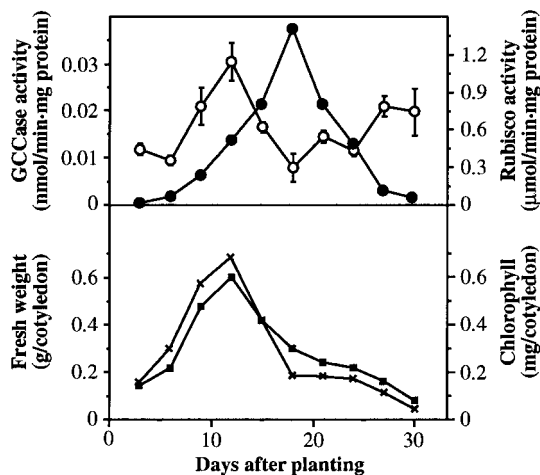


FIG. 7. Effect of cotyledon development on GCCase activity. Soybean cotyledons were harvested at the indicated times after planting and their fresh weight (■) and chlorophyll content (X) determined. GCCase (○) and Rubisco (●) activities were determined in extracts prepared from cotyledons.

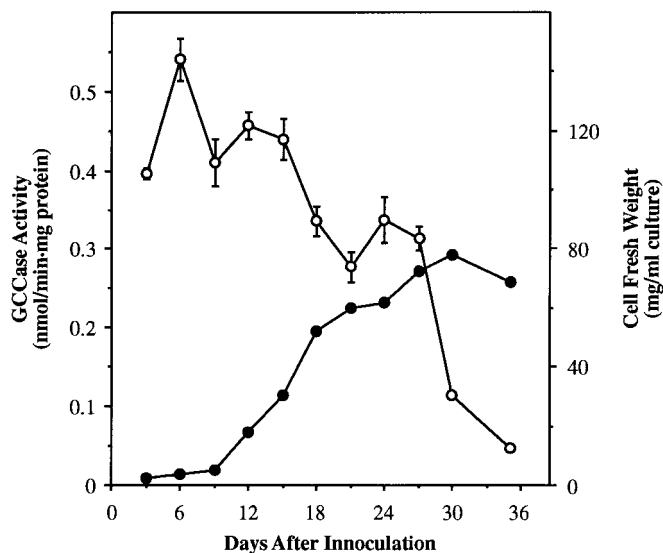


FIG. 8. Effect of cell growth on GCCase activity. A suspension of cultured carrot cells was inoculated into fresh medium, and cells were harvested at the indicated times. The growth of the culture was monitored by following the weight of cells (●), and GCCase activity (○) was determined in extracts of the cultured cells.

ular embryos), and subsequently increased during embryo development (Table IV).

DISCUSSION

This paper reports the discovery and initial characterization of GCCase in plants. The discovery of this biotin-containing enzyme in the plant kingdom identifies the biochemical function of the 122-kDa biotin-containing polypeptide, which was first observed by Western blot analyses of plant extracts probed with streptavidin (17). Previous to this report, GCCase had been reported only in a few bacterial species that are capable of using isoprenoids as a sole carbon source (1–5). The plant GCCase has structural and biochemical properties that are distinct from the GCCase of *P. citronellolis*, the only other GCCase that has been characterized (4, 7). The biotinylated subunit of the plant GCCase is 122 kDa, as opposed to 73 kDa for the bacterial enzyme. Although the preferred substrate for bacterial GCCase is geranyl-CoA, it will also carboxylate the γ -methyl group of the homologous C5 isoprenoid (methylcrotonyl-CoA) and C15 isoprenoid (farnesoyl-CoA) (4). In contrast, plant GCCase shows a stricter substrate preference and will only carboxylate geranyl-CoA but not methylcrotonoyl-CoA (we did not test farnesoyl-CoA).

Plants synthesize thousands of isoprenoids for diverse biological functions, such as growth regulation (i.e., abscisic acids, gibberellins, and cytokinins), photosynthesis (chlorophylls, plastoquinones, and carote-

noids), respiration (ubiquinones), membranes (sterols), insect attractants for pollination (carotenoids), and defense from biotic stresses (sesquiterpenes and diterpenes) (31–38). Despite indications of isoprenoid turnover in plants, the pathways of isoprenoid catabolism are largely unknown (34–37, 39, 40). Initial steps in the degradation of gibberellins, carotenoids, and phytol have been established, but the subsequent fates of these catabolites have not been determined (34, 37, 42–44). Croteau and colleagues have elucidated the early reactions of the catabolism of the cyclic isoprenoids, menthone (from *Mentha*) and camphor (from *Salvia*), reactions that generate acyclic isoprenoid products (36, 39, 40, 45, 46). Furthermore, radiotracer studies show that carbon derived from camphor and menthone is recovered as acetyl-CoA; although the specifics of this catabolism are not established, it was hypothesized that β -oxidation was involved (36, 39, 45).

We suggest the presence of GCCase in plants indicates that acyclic isoprenoids may be catabolized to acetyl-CoA by a mechanism analogous to that proposed for pseudomonads (Fig. 9 and Ref. 5). We envision the plastid to be the site of the initial catabolism of at least the plastid-localized isoprenoids (such as phytol and carotenoids). The operation of a GCCase pathway of isoprenoid catabolism in plants would necessitate coordination of several subcellular compartments: plastids [the location of phytol, carotenoids (33, 47), and GCCase (this paper)], microbodies [the major location of β -oxidation (49–51)], and mitochondria [the location of MCCase (52)]. The combined action of these processes would enable plants to degrade acyclic isoprenoids to acetyl-CoA. Furthermore, the proposed GCCase catabolic pathway indicates a function for MCCase in plants in addition to its involvement in leucine catabolism.

In *P. citronellolis*, GCCase is induced by exogenous isoprenoids, enabling these organisms to respond to

TABLE IV
GCCase Distribution among Developing Carrot Embryos

Plant tissue	GCCase activity ^a nmol (min mg protein) ⁻¹
Embryogenic cells (+ 2,4-D media)	0.274 ± 0.008
Small globular embryos	0.095 ± 0.009
Large globular embryos	0.110 ± 0.003
Small torpedo embryos	0.119 ± 0.001
Large torpedo embryos	0.15 ± 0.01
Mature embryos	0.205 ± 0.005

Note. GCCase activity was determined in extracts from the indicated developmental stages of carrot somatic embryos.

^a Data are means ± SD of three determinations.

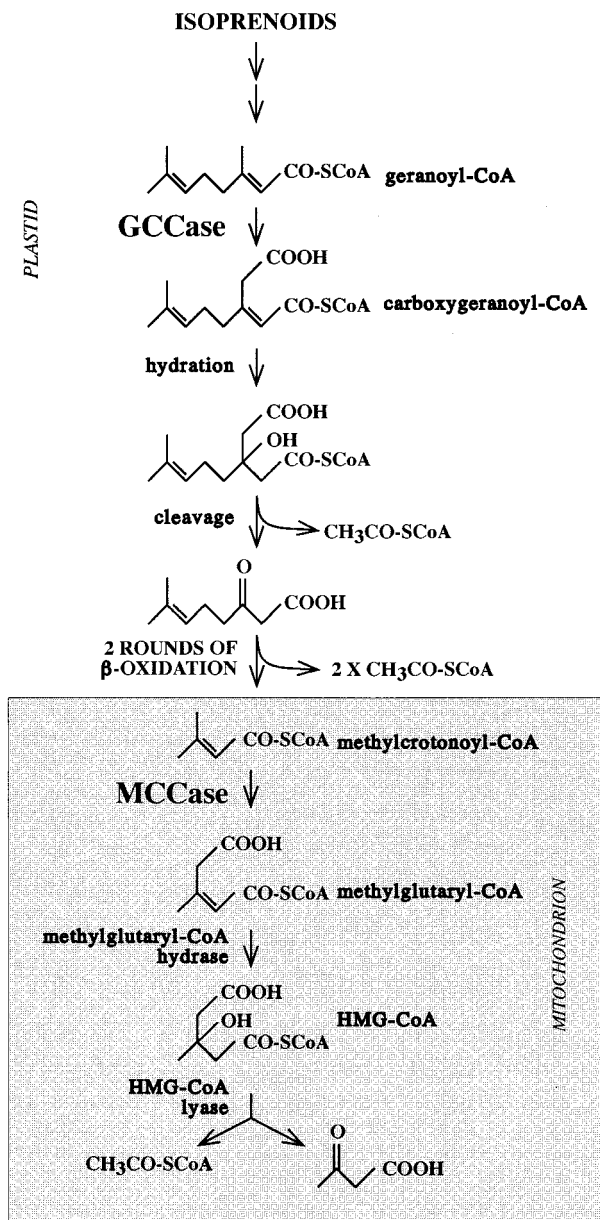


FIG. 9. Putative metabolic role of GCCase in the catabolism of isoprenoids. Geranyl-CoA, derived from the catabolism of isoprenoids, would be carboxylated in plastids by GCCase. The resulting carboxygeranyl-CoA could be further metabolized by sequential hydration and cleavage (by enzymes analogous to methylglutaryl-CoA hydratase and HMG-CoA lyase, respectively) to generate acetyl-CoA and 3-oxo-7-methyl-octenoate. This intermediate could then undergo two rounds of β -oxidation, which would generate two molecules of acetyl-CoA and methylcrotonyl-CoA. The latter could be converted to acetyl-CoA and acetoacetate by the sequential action of mitochondrial MCCase, methylglutaryl-CoA hydratase, and HMG-CoA lyase.

the availability of an alternative carbon source (5). In contrast, GCCase accumulates ubiquitously in plants, occurring in leaves, roots, seeds, and cultured cells. We

hypothesize this may reflect a continual recycling of many different types of isoprenoids in plants. Thus, the presence of GCCase in plants may indicate that the later steps of isoprenoid catabolism occur via a pathway requiring two biotin enzymes and three subcellular organelles. This catabolic pathway could serve both to remove no-longer-desirable isoprenoids and to re-coup carbon and energy from these molecules.

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