Biosynthesis of Lipase in the Scutellum of Maize Kernel*

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In the scutellum of maize kernel after imbibition, lipase activity increased rapidly, concomitant with the decrease in storage triacylglycerols. The enzyme activity peaked at day 6, but remained at the same level from day 6-10 when most of the triacylglycerols had been depleted. By \textit{in vitro} translation with extracted RNAs followed by immunoprecipitation, and by resolving the translation products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, lipase was found to be \textit{de novo} synthesized in postgermination. The enzyme was synthesized by RNAs extracted from free polyribosomes and not from bound polyribosomes. Both \textit{in vitro} and \textit{in vivo} synthesized lipase had the same \textit{M}, of 65,000 as resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as had the purified authentic enzyme; thus there was no appreciable co-or post-translational processing of the enzyme. Lipase-specific mRNA was present only between day 2-6 after imbibition. At day 6 when lipolysis was most active, more than 60\% of the lipase activity was recovered in the lipid body fraction and specifically associated with the organelle membrane. From day 6-10, the lipase activity gradually shifted from the lipid body fraction to other subcellular fractions, including the 10,000 \times g pellet, the 120,000 \times g pellet, and the 120,000 \times g supernatant. Lipase in these subcellular fractions was attributed to represent the enzyme associated with membrane ghosts of the lipid bodies which were fusing with the fragile cell vacuoles; such fusions were observed \textit{in situ} by electron microscopy.

During seed germination and seedling growth, the food reserve in the storage tissue is rapidly mobilized to support the growth of the embryonic axis. In oil seeds, the storage triacylglycerols are localized in organelle called lipid bodies. A lipid body has a matrix of triacylglycerol surrounded by a half-unit membrane of one phospholipid layer (1, 2). The triacylglycerols are hydrolyzed by lipase (EC 3.1.1.3) to fatty acids and glycerol, which are converted eventually to sugars (3, 4). In most seeds, lipase activity is absent before germination and appears in postgermination concomitant with the disappearance of storage triacylglycerols (5). Since lipase catalyzes the first step in a long metabolic pathway, its reaction may be rate-limiting to the whole pathway and thus a desirable target of developmental and metabolic regulation. The mechanism which controls the appearance of lipase activity is unknown. This appearance may be due to a \textit{de novo} synthesis of the enzyme during seedling growth or an activation of an inactive enzyme synthesized during seed maturation.

We have been using maize scutella as a model system to study seed lipase and lipid bodies (6-8). In this tissue, lipase activity is absent in the maturing and ungerminated seed, and appears 2 days after imbibition, concomitant with the disappearance of triacylglycerols. The lipase is tightly associated with the membrane of the lipid bodies during the active stage of lipolysis. The enzyme has a \textit{M}, of 65,000 as estimated by SDS-PAGE, and monospecific rabbit antibodies have been raised against the purified enzyme. The maize lipase is the only lipid body lipase that has been purified to apparent homogeneity.

In this paper, we report experimental results showing that the appearance of lipase activity in postgermination is due to a \textit{de novo} synthesis of the protein, that there is no co-or post-translational processing of the enzyme, and that the enzyme is synthesized on free and not bound polyribosomes. We also report findings on the fate of the lipase on the membrane of the lipid bodies after lipolysis.

\section*{EXPERIMENTAL PROCEDURES}

Materials

Kernels were collected 120 days after pollination (DAP). The inbred line of maize (\textit{Zea mays} L.), Mo 17, was obtained from the Illinois Foundation Seed Corporation (Champaign, IL). Kernels were soaked in running water for 24 h (designated as day 0) and allowed to germinate on moist vermiculite at 20°C in darkness. Scutella were isolated from seedlings of different ages. In the use of maturing kernels, the plants were grown in the field. When about 50\% of the maximal kernel lipids had been accumulated, the maturing kernels were collected.

\subsection*{Enzyme Preparations}

All operations were performed at 0-4°C. The grinding medium contained 0.15 M Tricine-KOH buffer (pH 7.5), 1 mM MgCl\(_2\), 10 mM KCl, 1 mM EDTA, 2 mM dithiothreitol, and 0.6 M sucrose. The tissues were collected, immersed in the grinding medium, chopped with a new razor blade, and then homogenized with a mortar and pestle. The slurry was filtered through a piece of Nitex cloth (Pentek, Elmsford, NY) of pore size 20 x 20 \(\mu\)m. The filtrate was referred to as the crude extract and was used to determine enzyme activities and the content of total lipids.

Subcellular Fractionation

Ten ml of the crude extract was placed in a centrifuge tube, and an equal volume of grinding medium containing 0.5 M instead of 0.6 M sucrose was layered on top. After centrifugation at 10,000 \(\times g\) for 15 min, the lipid bodies floated on top were removed with a spatula. The pellet (10,000 \(\times g\) pellet) was collected. The supernatant and the overlaying cushion were mixed and subjected to centrifugation at 120,000 \(\times g\) for 90 min in a Beckman L2-65B ultracentrifuge with a SW 65 rotor. The resulting supernatant (120,000 \(\times g\) supernatant) and pellet (120,000 \(\times g\) pellet) were obtained. The lipid bodies, the

* The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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Lipase Synthesis in Maize Scutellum

10,000 \times g pellet and the 120,000 \times g pellet were resuspended in grinding medium.

Enzyme Assays

Lipase—Lipase activity was determined by a colorimetric method (7, 9). Trilinolein from Nu Chek Prep Inc. (Elysian, MN) was used as the substrate. An emulsion of 25 mM trilinolein in 5% Gum Arabic was prepared by sonication at high intensity with a Bronwill Biosonik sonicator (VWR Scientific, San Francisco, CA) for 30 s. Lipolytic activity was measured in a 1-ml reaction mixture containing 0.1 M Tris-HCl buffer (pH 7.5), 5 mM trilinolein (from 0.2 ml of the substrate emulsion), 5 mM dithiothreitol, and lipase preparation. The reaction was carried out at 34 °C in a shaker-water bath. Aliquots of the reaction mixture were sampled at time intervals. The amount of fatty acids released was determined using L,5-diphenyloxazole (9).

Other Enzymes—Catalase activity was assayed spectrophotometrically at 24 °C by monitoring the disappearance of H_2O_2. Isocitrate lyase activity was assayed spectrophotometrically at 24 °C by measuring the appearance of glyoxylate-phenylhydrazine complex with phenylhydrazine (10).

Determination of Lipid Contents

One-hundred \( \mu l \) of crude extract was extracted with 4 ml of a chloroform/heptane/methanol mixture (4:3:2, v/v/v), and the acyl ester bonds in the organic solvent fraction were determined (11). Trilinolein was used as the standard, and the lipid content was expressed as \( \mu \)mol equivalent of acyl ester bond.

RNA Preparation

The scutella collected at various maturing and germinating stages were homogenized in 20 mM sodium-acetate buffer (pH 5.3), containing 10 mM EDTA and 7.5 M guanidinium chloride with a Polytron set at 6. The homogenate was centrifuged at 10,000 \( \times g \) for 10 min. Nucleic acids were precipitated from the 10,000 \( \times g \) supernatant with 0.7 volume of absolute ethanol. The pellet was resuspended in TES buffer (100 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 5 mM EDTA, and 0.1% SDS), phenol-chloroform extracted once, chloroform extracted once, and the nucleic acids were ethanol precipitated. DNA and low M_r RNAs were separated from high M_r RNAs by rinsing the ethanol precipitated nucleic acids with 2 M LiCl (12). The resulting high M_r RNAs were referred to as total RNAs. RNA preparations were stored in 70% ethanol at -20 °C.

Preparation of Free and Bound Polyribosomes

The separation of free and membrane-bound polyribosomes was carried out as described (13) with some modifications. All solutions were made up in the same buffer. The extraction medium consisted of 2.0 M sucrose, 200 mM Tris-HCl (pH 8.5), 10 mM MgCl_2, and 5 mM EDTA. After homogenization of scutella for 10 min, the homogenate was centrifuged at 10,000 \( \times g \) for 10 min. The supernatant was layered over the sample in sequence, and the tube was centrifuged at 27,000 rpm for 6 h in a Beckman SW 28 rotor. The transparent pellet at the bottom was resuspended in buffer B. The suspension was subjected to linear sucrose gradient centrifugation to resolve the polyribosomal fraction (referred to as the free polyribosomal fraction) as described in the preceding paragraph. The membrane fraction at the interface between 20 and 60% sucrose solutions from the 6 h, 27,000 rpm centrifugation was collected and diluted with 0.1 volume of 10% Nonidet P-40 solution. The solubilized polyribosomal fraction (referred to as the membrane-bound polyribosomal fraction) was obtained after the same linear sucrose gradient centrifugation. From the free and bound polyribosomes, RNAs were extracted as described in the preceding paragraph.

In Vitro Protein Synthesis

Total RNAs and polyribosomal RNAs prepared from maize scutella of different maturing and germinating stages were used to direct protein synthesis in vitro (14, 15). Two in vitro translation kits, wheat germ extract and rabbit reticulocyte lysate, were purchased from Bethesda Research Laboratories. They were used according to the instructions provided by the manufacturer, using [35S]methionine as the labeled amino acid. Preliminary experiments were performed to establish the range of RNA needed to produce a linear RNA-dependent translation.

Labeling of Proteins with [35S]Methionine in Vivo

The embryonic part (including scutellum and axis) was separated from 2.5-day-old seedling and fed with 5 \( \mu l \) of [35S]methionine (New England Nuclear; specific radioactivity was about 1000 Ci/mol) from the surface (originally facing the endosperm). The embryonic tissues were kept in the dark in a moist chamber and incubated at room temperature for 2 days. Scutella were separated from the seedlings and were used to obtain lipid bodies as described under "Subcellular Fractionation."

Immunoprecipitation

Immunoprecipitation was performed as described (16). The protein samples (containing about 1,000 dpm), of either in vitro translation products or in vivo labeled protein extracts, were solubilized in Solution A (150 mM NaCl, 10 mM Tris-HCl, pH 8.5, 10 mM EDTA, 1% Nonidet P-40), and then treated successively with preimmune serum and antibody-containing serum. In some tests to be described under "Results," the protein samples were denatured with SDS before incubation with preimmune serum. The protein sample was mixed with an equal volume of denaturing buffer consisting of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol in 62.5 mM Tris-HCl, pH 6.8. The mixture was heated for 2 min at 90 °C, and the SDS concentration in the mixture was diluted by adding 25 volumes of Solution A. Immunoprecipitation was then performed as described. Rabbit anti-lipase (maize) serum was prepared as described (6). Rabbit anti-\( \alpha \)-amylose (barley aleurone) serum was a gift from Dr. David Ho of Washington University, St. Louis; and rabbit anti-catalase (cotton cotyledon) serum was kindly provided by Dr. Richard Trelease of Arizona State University.

SDS-PAGE and Fluorography

Protein samples were analyzed by electrophoresis in 12.5 or 6% polyacrylamide/0.1% (w/v) SDS slab gel (17) of 1.5-mm thickness. After electrophoresis, the gel was fluorographed (18).

Electron Microscopy

Scutella (Zea mays L. KK 3732 P-12) were isolated from 6-day-old seedlings. The central part of the scutellum was dissected, submerged in one drop of fixative medium, and cut into small pieces. The fixative medium contained 2% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.4). After 20 h of fixation, the tissues were rinsed three times, of 15 min each, in 100 mM potassium phosphate buffer (pH 7.4), and postfixed 2 h with 2% OsO_4 in the same buffer. After rinsing three times with the same phosphate buffer, the tissues were dehydrated through a graded series of ethanol and then infiltrated and embedded in Spurr. Tissue sections were stained with 5% uranyl acetate and poststained with Reynolds' lead citrate (19, 20). The stained sections were examined in a Zeiss EM 9S-2 electron microscope.
RESULTS

Developmental Changes of Total Lipid and Enzyme Activities—Following imbibition of the maize kernel, the total lipid in the scutellum remained unchanged for 2 days and then disappeared rapidly, concomitant with the appearance of lipase activities (Fig. 1). The small change (10%) in the total lipid between day 0–2 is likely due to variations in sampling and analyses. The activities of catalase and isocitrate lyase, two enzymes known to participate in gluconeogenesis from lipids, appeared a day earlier than the lipase activity. The physiological significance of this earlier appearance of the two enzymes is unknown. The activities of all the three enzymes peaked at day 5–6. Thereafter, lipase activity remained at about the same level up to day 10, whereas catalase and isocitrate lyase activities dropped off rapidly.

In day 2–6 when lipolysis was most active, more than half of the lipase activity in the homogenate was recovered in the lipid body fraction (Fig. 1). Earlier findings show that lipase is a hydrophobic protein that binds tightly to the membrane of the lipid bodies (6). Beyond day 6 when most of the lipid had been depleted, lipase activity shifted from the lipid body fraction to other subcellular fractions, including the 10,000 × g pellet, the 120,000 × g pellet, and the 120,000 × g supernatant.

In Vitro Translation—RNAs were extracted from the scutella of maturing kernels and day 3 seedlings and were used to direct in vitro translation. The translation products were immunoprecipitated with antibodies raised against lipase, and the precipitates were analyzed by SDS-PAGE and fluorography. Initial experiments were performed to compare the translation efficiency of rabbit reticulocyte lysate and wheat germ extract. The reticulocyte system gave a higher incorporation of [35S]methionine into proteins and appeared to have a much better efficiency in translating high Mr RNAs (Fig. 2). The rabbit antilipase antibodies used in the experiment were raised against the native, enzymatically active maize lipase, which was prepared in sodium deoxycholate (6). These antibodies were able to precipitate the native enzyme in the total in vitro translation products but were unable to do so when the total translation products had been treated with SDS. In all subsequent experiments, the reticulocyte system was used, and the immunoprecipitation was performed in the absence of SDS.

Using the above in vitro translation system, lipase was found to be synthesized by RNAs extracted from the scutella of germinated but not maturing kernels (Fig. 3). The immunoprecipitated lipase had a Mr of about 65,000 as resolved by SDS-PAGE, which is in agreement with that of the purified enzyme (6). Lipase in the lipid bodies synthesized by in vivo labeling of the scutella of seedlings was compared with that synthesized by in vitro translation (Fig. 4). The two enzymes exhibited the same Mr of 65,000. The possible difference of the Mr of the two enzymes was further analyzed using 8% instead of 12.5% acrylamide gel. There was no appreciable difference in the Mr of the two enzymes when they were resolved by SDS-PAGE separately or in combination.

![Fig. 1. Changes in total lipid, lipase activity, lipase-specific mRNA (upper panel), isocitrate lyase activity, and catalase activity (middle panel), and the distribution of lipase activities in different subcellular fractions (lower panel) in the scutella of maize kernel during seedling growth. All contents and activities are expressed on a per scutellum basis. FFA, free fatty acid; P, pellet; SN, supernatant.](image-url)

![Fig. 2. Efficiency of reticulocyte lysate and wheat germ extract in supporting the in vitro translation of scutellum RNA and effect of SDS treatment of the translation products on subsequent immunoprecipitation. After in vitro translation using [35S]methionine and day 3 scutellum RNA, the translation products were treated with buffer alone (lanes 5–9) or buffer containing SDS (lanes 1, 2, 10, 11). Afterward, they were analyzed either directly (lanes 5, 7) or after immunoprecipitation with rabbit preimmune serum (lanes 1, 4, 8, 11) or anti-lipase antibodies (lanes 2, 3, 5, 10). They were subjected to SDS-PAGE, and the gel was fluorographed. Mr markers (lane 6) are indicated on right.](image-url)
Synthesis of Lipase on Free Polyribosomes—Whether lipase was synthesized on free or bound polyribosomes was assessed. The two populations of polyribosomes were isolated, and their RNAs were extracted and used for in vitro translation. The identity of the two populations of polyribosomes was confirmed by their ability to synthesize catalase and α-amylase, enzymes known to be synthesized by free polyribosomes in mammalian systems (21), and by bound polyribosomes in barley (22), respectively. As shown in Fig. 5, catalase was synthesized predominantly by RNA from the free polyribosomal fraction, whereas α-amylase was synthesized exclusively by RNA from the bound polyribosomal fraction. The detected Mₐ of catalase was about 55,000, and that of amylase was about 43,000 (two isozymes); these values are agreeable with those reported earlier (21, 22).

Lipase was predominantly synthesized by RNA extracted from the free polyribosomal fraction (Fig. 5). There was a trace of lipase synthesized by RNA extracted from the bound polyribosomal fraction. We attribute this trace of lipase to have been synthesized by RNA from free polyribosomes trapped in the bound polyribosomal fraction. In line with this attribution, a trace of catalase was also synthesized by RNA from the bound polyribosomal fraction. In the media for the preparation of the two populations of polyribosomes, high Mg²⁺ concentrations were used which led to clumping of organelles. Similar trapping of free polyribosomes in bound polyribosomal fractions has been observed in several mammalian systems (23–25). In our preparation, reduction of the Mg²⁺ and EGTA concentrations in the preparative media to ½ and ⅔ of the original concentrations resulted in poor recovery of bound polyribosomes.

Synthesis of Lipase in Seedlings of Different Ages—RNAs prepared from the scutella of seedlings of different ages were used to direct in vitro translation. Immunoprecipitation of the translation products revealed that lipase-specific mRNA increased after day 2, peaked at day 5, and thereafter disappeared rapidly (Fig. 1). The increase in lipase-specific mRNA from day 2–5 preceded slightly the increase in lipase activity. Between day 6–10 when little lipase-specific mRNA was present, the increase was marked by a rise in lipase activity in vivo on bound polyribosomes [lanes 3, 5, 6].

In vitro translation of RNAs extracted from scutella of maturing or germinated (day 2) kernels. After in vitro translation using [³⁵S]methionine and scutellum RNA, the translation products were analyzed either directly (lanes 1, 4) or after immunoprecipitation with rabbit preimmune serum (lanes 3, 6) or anti-lipase antibodies (lanes 2, 5). They were subjected to SDS-PAGE, and the gel was fluorographed. Mₐ markers (lane 7) are indicated on right.

Comparison of lipase synthesized in vitro and in vivo translation. In vitro translation using [³⁵S]methionine and scutellum RNA (day 3) and in vivo translation by applying [³⁵S]methionine to intact scutella (day 3) were performed. The translation products were analyzed either directly (lanes 1, 4) or after immunoprecipitation with rabbit preimmune serum (lanes 3, 5) or anti-lipase antibodies (lanes 2, 6). They were subjected to SDS-PAGE (12.5% acrylamide), and the gel was fluorographed. The immunoprecipitated lipase from in vivo and in vitro translation products were analyzed separately (lanes 7, 8) or in combination (lane 9) by SDS-PAGE (8% acrylamide), and the gel was fluorographed. Mₐ markers are indicated on right.

In vitro translation of RNAs from free and bound polyribosomal fractions prepared from scutella of day 3 seedlings. After in vitro translation with [³⁵S]methionine and RNAs from total (f), free (l), or bound (b) polyribosomal fractions, the translation products were analyzed directly (lanes 10–12) or after immunoprecipitation with rabbit serum containing anti-catalase (lanes 1–3), anti-α-amylase (lanes 4–6), and anti-lipase (lanes 7–9) antibodies. They were subjected to SDS-PAGE, and the gel was fluorographed. Mₐ markers are indicated on right.
ent, the unaltered lipase activity was apparently due to left-over lipase molecules synthesized before day 6.

Fate of Lipase at Late Stages of Seeding Growth—At a late stage of seeding growth (day 6-10) when most of the lipid had been depleted, lipase activity remained at the same level even though catalase and isocitrate lyase activities dropped off rapidly (Fig. 1). In subcellular fractionation of the tissues of increasing ages, lipase activity associated with the lipid bodies gradually shifted to the 10,000 x g pellet, the 120,000 x g pellet, and the 120,000 x g supernatant. It is likely that the membrane, together with the associated lipase, of the lipid bodies after the matrix lipid had been depleted remained inside the cells as "ghosts" of the organelles, or joined with the membrane of other fragile organelles such as the vacuoles. In the latter aspect, the very fragile vacuoles and fusing lipid body-vacuoles would be lysed in our subcellular fractionation by differential centrifugation, leading to the recovery of the transparent regions, probably of aqueous nature, in the matrix of the membrane of other fragile organelles such as the vacuoles. The fusion generally occurred at the lipid membrane of the lipid body, together with the vacuoles. The fusion generally occurred at the lipid membrane adjacent to the electron transparent regions.

Electron microscopic observation of the scutellum cells at a late stage of lipolysis (day 6, see Fig. 1) confirmed the latter speculation (Fig. 6). Many lipid bodies contained electron transparent regions, probably of aqueous nature, in the matrix in substitution of the depleted lipid. The lipid bodies partially depleted of matrix lipid tended to fuse with one another or with the vacuoles. The fusion generally occurred at the lipid body membranes adjacent to the electron transparent regions.

**DISCUSSION**

In postgerminative growth of maize kernel, the appearance of lipase activity in the scutellum is due to a de novo synthesis of the lipase protein. The protein is synthesized on free and not on bound polyribosomes. After synthesis, the enzyme migrates and attaches itself to the membrane of the lipid bodies. There is no appreciable co- or post-translational processing of the protein. At a late stage of seedling growth when most of the lipid has been depleted, lipase synthesis stops. The membrane of the lipid body, together with the still-active lipase, fuses with the membranes of other lipid bodies or of the vacuoles. During the fusion, the monolayer of phospholipids of the lipid body membrane, by virtue of its hydrophobicity on the side facing the original triacylglycerol matrix, should undergo rearrangement to form the double layer of phospholipids of the vacuole membrane.

Lipase synthesized on free polyribosomes migrates and attaches itself to the membrane of the lipid bodies but not to the membranes of other organelles. This specific binding should be due to recognition signals residing in both the enzyme and the lipid body membrane. The native lipase has a highly intrinsic conformation and contains specific antigenic properties which are destroyed by SDS treatment. It is unlikely that the recognition signal on the lipid body membrane resides in the phospholipids. The membrane does not contain uncommon phospholipids, and it is generally agreed upon that the common phospholipids do not have sufficient signals for specific protein binding. It is more likely that proteins on the membrane of the organelles provide the recognition signals. Membrane proteins unique to the lipid bodies of no known function are present in maize scutella (26). Whether or not they indeed provide the recognition signal for lipase binding remains to be seen.

**REFERENCES**