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Lipase in the Lipid Bodies of Corn Scutella during Seedling Growth[1]

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ABSTRACT

In the scutella of corn (*Zea mays*), lipase activity is absent in ungerminated seeds and increases during seedling growth. At the peak stage of lipolysis, about 50% of the lipase activity is recovered in the lipid body fraction after flotation centrifugation. The lipase is tightly bound to the lipid bodies, and resists solubilization by repeated washing with buffers or NaCl solutions. Isolated lipid bodies undergo autolysis of internal triacylglycerols, resulting in the release of fatty acids. After the triacylglycerols in isolated lipid bodies have been extracted with diethyl ether, the lipase is recovered in the membrane fraction. The lipase has an optimal activity at pH 7.5 in the autolysis of lipid bodies, or on trilinolein or *N*-methylindoxylmyristate. Of the various acylglycerols examined, the enzyme is active only on acylglycerols of linoleic and oleic acids which are the major fatty acid constituents of corn oil. The activity is not greatly affected by NaCl, $CaCl_2$, or pretreatment of the enzyme with *p*-chloromercuribenzoate or mersalyl, and detergents abolish the activity. The enzyme hydrolyzes trilinolein completely to fatty acids; during the course of reaction, there is little accumulation of di- or mono-linolein.

Most seeds contain intracellular triacylglycerols as major food reserve for germination. During early seedling growth, the triacylglycerols in the storage tissues are mobilized to support the growth of the storage tissues and the embryonic axis. The initial step in the mobilization of triacylglycerols is their hydrolysis to fatty acids and glycerol. Since this hydrolysis is the first step in a long gluconeogenic pathway, its activity may be important in the regulation of the pathway. Surprisingly, there is little information on the lipase involved in the triacylglycerol hydrolysis in oil seeds. Only the lipase from castor bean has been studied to a great extent (12–14). The castor bean lipase has an optimal activity at acidic pH 4.1. It is already present at a maximal activity in the ungerminated seeds, and during seedling growth its activity decreases concomitant with the decrease in storage triacylglycerols. The castor bean acid lipase is unique among oil seeds of diverse species, since none of the other oil seeds examined contains a high activity of lipase in the ungerminated seeds (4, 6).

The storage triacylglycerols are localized in the organelles called lipid bodies (oleosomes, oil bodies, spherosomes) which are surrounded by a membrane (7, 13, 19). The lipid body should be a prime candidate for the subcellular location of lipase, since for lipolysis to occur the enzyme has to come into contact with the substrate. In castor bean, the enzyme is localized on the membrane of the lipid bodies (13). However, other than the unique castor bean, there is no information on the occurrence of lipase in the lipid bodies of oil seeds. On the contrary, lipid bodies isolated from peanut (7), pine (3), and soybean (8) from ungerminated and germinated seeds do not contain lipase activity. In addition, lipase activities were detected in the soluble fraction of pine megagametophyte (3) and the glyoxysomes of soybean (8).

In this report, we present findings on the lipase in the scutella of corn. The corn lipase is present in the lipid bodies of germinated but not ungerminated seeds. It has an optimal activity at neutral pH values, and is highly specific for the native corn triacylglycerols. The current findings, together with similar findings on rapeseed, mustard seed, and cotton seeds (to be reported elsewhere), establish a pattern of characteristics of oilseed lipase.

MATERIALS AND METHODS

Plant Materials. Unless otherwise stated, inbred corn (*Zea mays*) MO-17 obtained from Illinois Foundation Seed Corporation (Champaign, IL) was used. Hybrid corn EK 3732-F12 was obtained from Pioneer Seed Company (Johnston, IA). The seeds were soaked in water for 24 h at room temperature (24°C) and allowed to germinate in moist paper towels at 29°C in darkness. In germination studies, seeds were selected for uniformity before use, and seedlings were selected by both chronological age and hypocotyl length.

Preparation of Extracts and Organelles. The scutella were removed carefully from the embryonic axis and endosperm. In germination studies, 10 scutella of each age were used. The scutella were washed three times with distilled H_2O and ground in 10 ml of grinding medium with a mortar and pestle. The grinding medium contained 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, and 0.15 M Tricine buffer adjusted to pH 7.5 with KOH. The homogenate was filtered through a piece of Nitex cloth (Petko, Elmsford, NY) of pore size 20 × 20 μm^2. The filtrate was used to determine the content of enzyme activities and other components.

In the preparation of subcellular organelles, 30 scutella were chopped with a razor blade in 30 ml of grinding medium, and ground gently with a mortar and pestle. After filtration, 15 ml of the filtrate was placed in one 40-ml centrifuge tube, and 15 ml of grinding medium containing 0.5 M instead of 0.6 M sucrose was layered on top. After centrifugation at 10,000*g* for 10 min, the lipid bodies floated to the top and were removed with a spatula. The pellet (10,000*g* pellet) was collected, The supernatant and the floating cushion were mixed, and an aliquot was centrifuged at 100,000*g* for 2 h in a Beckman L-2-65B ultracentrifuge with Spinco rotor SW 65. The resulting supernatant (100,000*g* supernatant) and pellet (100,000*g* pellet) were obtained. The lipid bodies, the 10,000*g* pellet, and the 100,000*g* pellet were resuspended in grinding medium.

In the preparation of lipid body membranes, the isolated lipid bodies were resuspended in grinding medium. The triacylglycerols were extracted three times with diethyl ether. The trace

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amount of diethyl ether was evaporated under a stream of N_2.

Assays. Lipase activity was measured by a colorimetric method. The fatty acids released were converted to copper soaps and quantitated using 1,5-diphenylcarbazide as the color reagent (11). The 1-ml reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.5) or other buffers as stated, 5 mM DTT, 2.5 mM substrate, and enzyme preparation. The substrates were emulsified in 5% gum acacia (Specialty Chemicals, Morristown, NJ) for 30 s at high speed with a Bronwill Biosonik IV ultrasonic generator (VWR Scientific, San Francisco, CA) fitted with a microprobe.

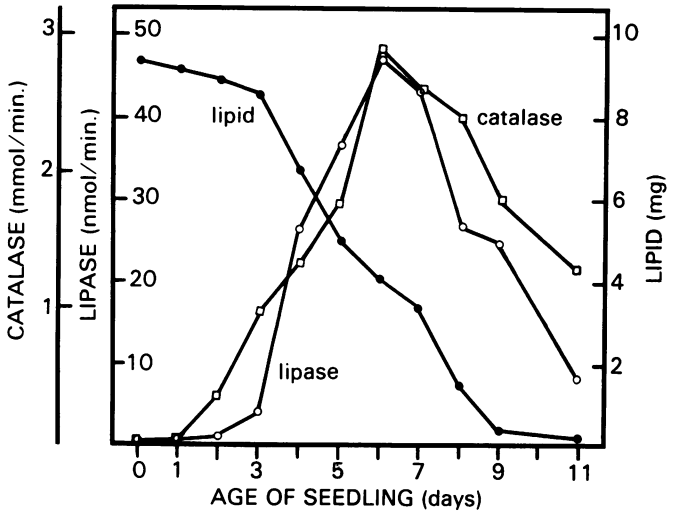

FIG. 1. Changes in lipid and catalase activity in the total homogenate and lipase activity in isolated lipid bodies of the scutella of corn seedlings. The contents are expressed on a per seed basis.

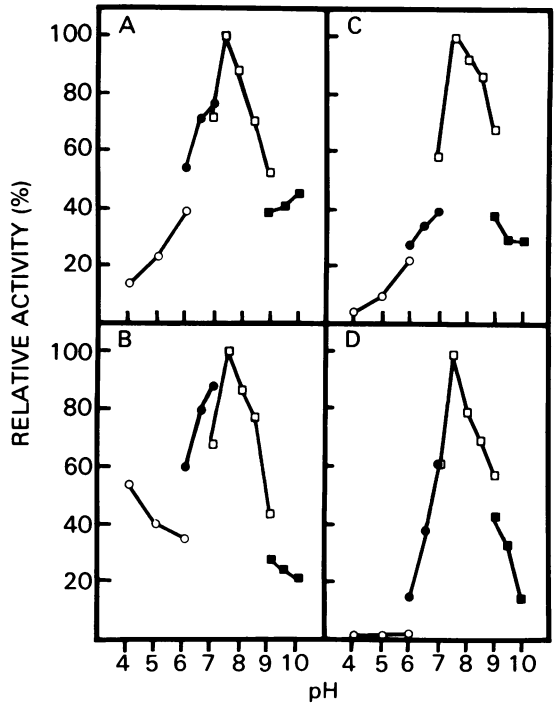

FIG. 2. Effect of pH on lipase activity in corn preparations. Buffers were succinate-HCl (4–6), imidazole-HCl (6–7), Tris-HCl (7–9), and glycine-NaOH (9–10). A, Lipase activity in crude homogenate on trilinolein. B, Autolytic activity of isolated lipid bodies in releasing fatty acids from endogenous lipids. C, Lipase activity in isolated lipid-body membrane fraction on trilinolein. D, Lipase activity in isolated lipid-body membrane fraction on *N*-methylindoxylmyristate.

To obtain a good dispersion of monopalmitin, dipalmitin, trierucin, tripalmitin, and tristearin, the lipids were dissolved in a few drops of chloroform and then added to the substrate solution before emulsification; after emulsification, the chloroform in the substrate emulsion was evaporated by a stream of N_2. The reaction was carried out at 34°C in a shaker-water bath, and the fatty acid contents in aliquots of the reaction mixture were measured. For each assay, two enzyme concentrations and five appropriate time intervals of sampling of the reaction mixture were carried out to ensure that linear kinetics were obtained. Standard curves of linoleic acid, palmitic acid, and erucic acids were constructed, and standard curves of other fatty acids followed those published (11) using palmitic acid as a common reference.

In TLC of the reaction mixture, samples were taken at time intervals, and analyzed for the content of substrates and reaction products. The lipids in the samples were extracted with chloroform methanol 2:1 (v/v), and spotted onto a TLC plate coated with 250 μm of Silica Gel G (Brinkman Instruments, Inc., Westbury NY). The plate was developed on 50:50:1 (v/v/v) hexane:diethyl ether:acetic acid, and allowed to react with iodine vapor. An estimate of the iodine-reactive products on the plate was performed using a densitometer (model 52-C; Photovolt Corp., New York) (18). Standards of varying amount of tri-, di-, and mono-linolein, and linoleic acid were run on a TLC plate, and the density of the various iodine-reactive lipid spots on a single plate appeared to be proportional to their respective amounts of linoleic acid moiety.

The activities of catalase, Cyt *c* oxidase, and Cyt *c* reductase were assayed spectrophotometrically as described earlier (8). Acyl hydrolase activity on *N*-methylindoxylmyristate was assayed fluorimetrically (10).

The lipids in crude cotyledon homogenates were extracted by the Bligh and Dyer method (1). The amount of lipid was determined by drying an aliquot of the chloroform extract in a vacuum oven over night and weighing the lipid residue.

Tripalmitin, tristearin, 1,3-dilinolein (containing 3–5% 1,2-isomer), 1,3-dipalmitin (containing 1–2% 1,2-isomer), dierucin (30% 1,3- and 70% 1,2-isomers), diolein (containing 85% 1,3-isomer), monolinolein, monoolein, monoerucin, and monostearin were obtained from Sigma. Trilinolein, tribehenin, triolein, trierucin, triarachidin, distearin (unspecified isomer composition), and monopalmitin were obtained from NU Check, Prep. Inc. (Elysian, MT). All the monoacylglycerols were of unspecified isomeric composition. *N*-methyl-indoxylmyristate was obtained from ICN Pharmaceuticals (Cleveland, OH). The substrates were checked for purity by the TLC system described above using hexane:diethyl ether:acetic acid as the developing solvent.

RESULTS AND DISCUSSION

Lipolysis and Lipase in the Scutella of Corn Seedlings. In early seedling growth, the lipids in the scutella disappeared rapidly after day 3 (Fig. 1). This disappearance was concomitant with the appearance of catalase, which is a marker of glyoxysomes that are essential for gluconeogenesis. At day 6, the total scutellum homogenate was assayed for lipase activities at various pH values. Using trilinolein as the substrate, the lipolysis was most active at pH 7.5 (Fig. 2A).

Using trilinolein as a substrate to measure lipase activity at pH 7.5, the subcellular location of the enzyme was assessed in various subcellular fractions obtained by differential centrifugations (Table I). Catalase and Cyt oxidase were assayed as markers of glyoxysomes and mitochondria, respectively, and NAD-Cyt reductase was assayed as a marker of mainly ER and mitochondrial membrane. About 50% of the lipase activity was recovered in the lipid bodies, and the rest was distributed among other sub-

Table I. *Localization of Lipase in Various Subcellular Fractions Separated by Differential Centrifugations of the Homogenate of Scutella from 6-Day-Old Corn Seedlings*

Enzyme activities are expressed on per seed basis.

Fractions	Lipase	Catalase	Cyt *c* Oxidase	Cyt *c* Reductase
	nmol/min	*μmol/min*	*nmol/min*	
Lipid bodies	40 (51)	0 (0)	0 (0)	0 (0)
Pellet (10,000*g*)	15 (19)	1137 (46)	5.9 (88)	5.2 (30)
Pellet (100,000*g*)	12 (15)	103 (4)	0.6 (9)	10.1 (59)
Supernatant (100,000*g*)	12 (15)	1238 (50)	0.2 (3)	1.8 (11)

Table II. *Effect of Washing on the Association of Lipase Activity with Lipid Bodies or Lipid-Body Membranes Isolated from Corn Scutella*

Isolated lipid bodies were washed with grinding medium containing 0.6 M sucrose and recovered after a flotation centrifugation using an overlayed cushion of grinding medium containing 0.5 M sucrose. In the third wash, the grinding medium also contained 0, 0.05, 0.2, or 2 M NaCl. In a separate experiment, the lipid-body membrane fraction obtained after ether extraction was concentrated in a pellet after a centrifugation of 150,000*g* for 90 min; it was washed with grinding medium containing 0, 0.05, 0.2, or 2.0 M NaCl, or 40% ethanol, and centrifuged at 150,000*g* for 90 min to recover the membrane pellet. The percent of lipase activity associated with the recovered lipid bodies or lipid-body membranes after washing are shown. Relative activities of 100 represent 12 and 16 nmol/h for the isolated lipid bodies and lipid-body membranes, respectively.

Treatments	Activity (relative)	
Isolated lipid bodies	100	
First wash with grinding medium	94	
Second wash with grinding medium	88	
Third wash with grinding medium	79	
		100
Third wash using medium plus 0.05 M NaCl		80
Third wash using medium plus 0.2 M NaCl		83
Third wash using medium plus 2.0 M NaCl		95
Lipid body membrane washed with medium containing:		
0 M NaCl	100	
0.05 M NaCl	100	
0.2 M NaCl	95	
2.0 M NaCl	95	
40% Ethanol	70	

Table III. *Effect of Various Reagents on the Activity of Corn Lipase in the Lipid-Body Membranes*

Trilinolein was used as the substrate. Relative activity of 100 represents 18 nmol/h.

Treatments	Relative Activity
None	100
NaCl (0.05 M)	124
NaCl (0.2 M)	108
NaCl (0.4 M)	103
$CaCl_2$ (10 mM)	77
$CaCl_2$ (50 mM)	53
EDTA (1 mM)	45
EDTA (10 mM)	0
Na_3PO_4 (5 μM)	78
Na_3PO_4 (20 μM)	57
SDS (2 mM)	0
SDS (20 mM)	0
Triton X-100 (0.1%)	0
Triton X-100 (1%)	0
p-Chloromercuribenzoate (0.1 mM)[a]	102
Mersalyl (0.2 mM)[a]	93
Omission of DTT (5 mM)	98

[a] The enzyme was incubated with sulfhydryl reagent for 10 min before the enzyme assay.

Table IV. *Hydrolysis of Various Acylglycerols by Corn Lipase in the Lipid-Body Membranes from Inbred MO-17 or Hybrid EK 3732-F12*

Relative activities of 100 represent 24 and 28 nmol/h for the enzymes from varieties MO-17, and EK 3732-F12, respectively.

Substrates	Hydrolytic Activity (Relative)	
	MO-17	*EK 3732-F12*
Trilinolein	100	100
Triolein	38	49
Tristearin	0	0
Tripalmitin	0	0
Trierucin	0	0
Triarachidin	0	Not tested
Tribehenin	0	0
Dilinolein	42	31
Diolein	50	50
Distearin	0	Not tested
Dipalmitin	0	0
Dierucin	0	0
Monolinolein	104	72
Monolein	88	94
Monostearin	0	0
Monopalmitin	8	18
Monoerucin	0	0

cellular fractions. In this subcellular fractionation, about 50% of the catalase was recovered, presumably in intact glyoxysomes, in the 10,000*g* pellet.

The lipase activity in lipid bodies increased during seedling growth, parallel to the increase of catalase activity (Fig. 1). At day 6, the lipase activity was 47 nmol fatty acid released/min/scutellum at 34°C, and is equivalent to a complete realease of fatty acids from 21 mg trilinolein/d. This activity at 34°C is much higher than the *in vivo* rate of lipolysis at 29°C, which is roughly 1.4 mg/d from day 3 to day 9 (Fig. 1). The lipid bodies isolated from germinated seed (day 5–6), but not from ungerminated seed, underwent autolysis of internal triacylglycerols, resulting in the release of fatty acids. The maximal autolytic activity also occurred at pH 7.5 (Fig. 2B).

Association of Lipase with Lipid-Body Membrane. The lipase was tightly associated with the lipid bodies. It resisted solubilization after repeated washing with grinding medium or NaCl solutions (Table II). The membrane of the isolated lipid bodies was obtained after the triacylglycerols had been extracted with diethyl ether. This ether extraction procedure has been used successfully to obtain the membrane of lipid bodies from castor

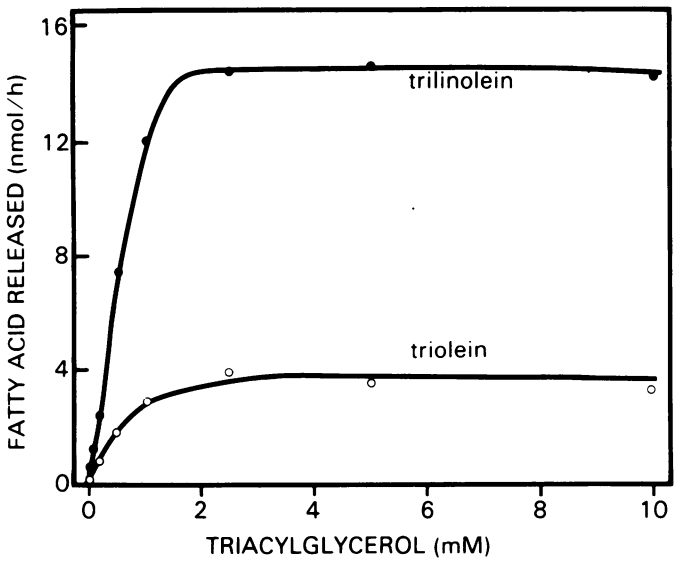

FIG. 3. Activity of lipase in isolated lipid-body membrane fraction on trilinolein or triolein at increasing concentrations. There was no activity on tristearin at a similar range of concentrations.

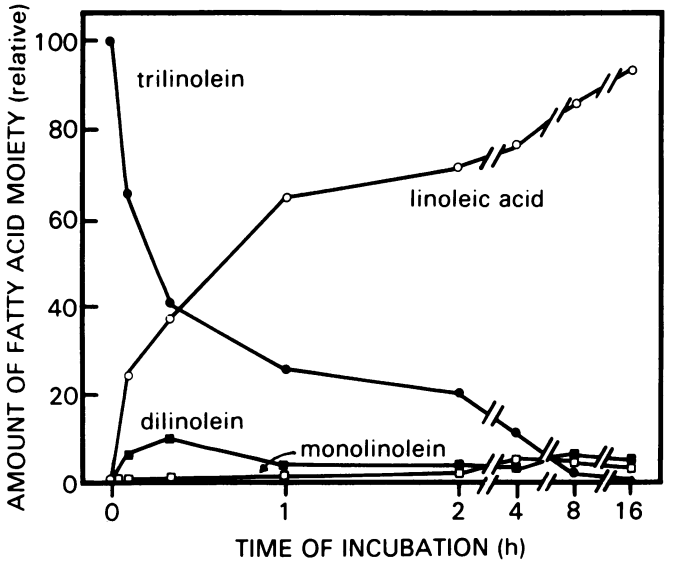

FIG. 4. Time course of hydrolysis of trilinolein by lipase in isolated lipid-body membrane fraction. The dilinolein represents roughly equal amounts of the 1,2- and 1,3-isomers.

bean (13), jojoba (9), and soybean (8). Earlier observation with the electron microscope showed that the corn lipid body membrane fraction obtained after ether extraction maintained the membrane integrity (17). The lipase was still tightly associated with the isolated membrane fraction, and resisted solubilization by 0.05, 0.2, and 2.0 M NaCl solutions or 40% ethanol (Table II).

Properties of Lipid-Body Lipase. The properties of lipase in the membrane fraction were studied. The lipase had an optimal activity at pH 7.5 on trilinolein (Fig. 2C), or *N*-methylindoxyl-myristate (Fig. 2D). Its activity was slightly enhanced in the presence of 0.05 M NaCl, and was reduced by $CaCl_2$, EDTA, and phosphate (Table III). Its activity was completely abolished by Triton or SDS. The enzyme was not inactivated by *p*-chloromercuribenzoate or mersalyl. The corn lipase is thus different from the castor bean acid (14) and alkaline lipase (10), peanut lipase (15), and wheat germ lipase (15), which are strongly inhibited by sulfhydryl group modifiers.

The lipase was active on trilinolein and triolein, and was totally inactive on tristearin, tripalmitin, trierucin, triarachidin, and tribehenin (Table IV). Its activity on increasing amounts of trilinolein and triolein prepared under identical conditions is shown in Figure 3. There was no activity on tristearin at a similar range of concentrations. The enzyme was also active on di- and mono-linolein and olein, and was inactive on di- and monopalmitin, stearin, and erucin (Table IV). Similar substrate specificity was also observed with the lipase prepared by a similar method from hybrid corn EK 3732-F12 (Table IV). The above substrate specificity is of physiological significance, since corn triacylglycerols contain about 60% linoleic acid and 30% oleic acid (5). Furthermore, its lack of activity on trierucin is in direct contrast to the rape seed or mustard seed lipase which is most active toward trierucin (unpublished). The two *Brassica* seeds contain erucic acid as the major fatty acid constituents of the storage triacylglycerols.

The kinetics of trilinolein hydrolysis by the lipase are shown in Figure 4. Trilinolein was hydrolyzed rapidly to linoleic acid. There was an accumulation of only a small percentage of dilinolein and monolinolein. This is in contrast to the kinetics of triacylglycerol hydrolysis by pancreatic lipase (2). The pancreatic lipase is much less active on monoacylglycerols than on triacylglycerols; in an *in vitro* hydrolysis of triacylglycerols, monoacylglycerols accumulate to a high percentage before they are eventually hydrolyzed to fatty acids. This contrast apparently reflects the difference in the physiological role of the corn and mammalian enzymes. The corn lipase hydrolyzes triacylglycerols to three fatty acids for β-oxidation in the glyoxysomes. The pancreatic lipase hydrolyzes triacylglycerols to two fatty acids and one monoacylglycerols for intestinal absorption.

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