Lipolytic Activity of California-Laurel (*Umbellularia californica*) Seeds

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ABSTRACT: The lipid content of dormant mature seeds of the California-laurel (*Umbellularia californica*) was 74% (mass basis) and decreased upon germination, reaching 43% 5 wk after germination. Dormant seeds contained only barely detectable lipase activity. Lipase activity rose upon germination, peaking 2 to 3 wk after the onset of visible germination. The combined addition of three detergents, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (1 mM), taurocholic acid (1 mM), and Tween-20 (0.05%, vol/vol) during homogenization increased the lipolytic activity of total seedling homogenates by about 60%. Following centrifugation of homogenates from seedlings of various ages, *ca.* 80% of the recovered lipolytic activity was located in the fat-free supernatant, with the remainder in the floating fat pad. The crude seed lipase did not show hydrolytic specificity for glycerol esters of lauric acid (the predominant fatty acid of the seed triglycerol): comparable lipolytic activities were seen toward olive oil, trilaurin, tripalmitin, and tristearin. Maximal lipolytic activity occurred at pH 8.5. This activity was stable over the pH range 6 to 9, and unstable at >40°C in a fashion that suggested the presence of multiple enzymes with different substrate specificities. Two lipolytic species, one of which showed some selectivity toward lauric acid esters, were partially separated from one another by ion-exchange chromatography.

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KEY WORDS: Biosynthesis, California-laurel, enzyme, Lauraceae, lipase, *Umbellularia californica.*

Prior to the production of the first leaves, a developing seedling relies upon stored reserves for energy and catabolic building blocks. In many seeds, triacylglycerols are a significant form of storage of these reserves. Their metabolism is initiated by lipases (glycerol ester hydrolase, EC 3.1.1.3), which hydrolyze the acylglycerols, ultimately releasing free fatty acids and glycerol. Lipases are produced by animals and microorganisms as well as plants. They have been detected in the food-reserve tissues of growing seedlings of many plants, especially those that contain large amounts of triacylglycerols (1,2). Various lipases have been developed and used in numerous applications for enzymatic catalysis (3). The biochemical characterization of seed lipases is important in understanding their roles within the developing seedling and in developing these enzymes for use as applied catalysts.

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Although dormant seeds of castor (4), oat (5), rice (2), wheat (2), *Nigella* (6), and hazel (7) contain detectable levels of lipolytic activity, it is more commonly the case that lipase activity is absent in dormant seed and appears early in germination.

A correlation has in some instances been found between the fatty acid composition of the storage lipids of a seed and the fatty acid selectivity of its lipolytic activity. For example, corn lipase is primarily active toward oleic and linoleic acids, the major constituent fatty acids of corn oil (8). Castor and rape lipases exhibit highest activity toward the predominant fatty acid constituents of these seeds, i.e., ricinoleic and erucic acids, respectively (8). In these cases the activities are only two- to threefold greater toward the predominant fatty acid than toward others.

In many other cases, the seed storage lipid is enriched in a particular fatty acid, and the lipase produced by that seed displays a corresponding substrate specificity. For example, the seed lipids of the American elm (*Ulmus americana*) are composed almost entirely of tricaprin, and elm seed lipase hydrolyzes tricaprin between 16 and greater than 100 times more readily than triacylglycerols of other fatty acids (8). Similarly, *Vernonia galamensis* seeds are rich in triacylglycerols containing vernolic (12,13-epoxy-9-octadecenoic) acid, and contain a lipase that hydrolyzes esters of this fatty acid 33 times faster than esters of oleic acid, the corresponding nonepoxy fatty acid (9). Examples such as the above suggest that lipases with high substrate specificities toward particular fatty acids may be found in seeds whose oils are rich in individual fatty acids.

California-laurel (*Umbellularia californica* Nutt., Lauraceae, also known as California bay laurel and by several other names) is a broad-leaved evergreen that matures either as a shrub or tall forest tree. It grows naturally in the extreme western United States of America from mid-Oregon southward to the Mexican border (10,11). The ovoid mature seeds of the species are slightly less than 2 cm in length and surrounded by a thin shell. The seeds are rich in triacylglycerols, which have been reported to contain between 43 and 70% lipid by weight (12–14). Lauric acid (12:0) is the primary component of these triacylglycerols, generally constituting 60 to 70% of the lipid-linked fatty acids, with capric acid (10:0) making up most of the balance (12–15). In view of the high seed lipid content and the predominance of lauric acid in the seed triacylglycerols of *U. californica,* we undertook an

investigation of the presence of lipase in the mature and germinating seeds of this species and an examination of the fatty acid selectivity of this activity. While this work was underway, Hellyer *et al.* (15) reported the presence in germinating *U. californica* seeds of lipolytic activity that displayed a threefold preference for acylglycerol esters of saturated fatty acids 8 to 12 carbons in length relative to unsaturated C18 fatty acids.

MATERIALS AND METHODS

Materials. Umbellularia californica seeds were obtained from Mistletoe Quality Seeds (Goleta, CA). Phaltan and Benomyl fungicides were provided by Ortho (San Francisco, CA) and Rockland Corp. (West Caldwell, NJ), respectively. Detergents, ion exchange resin, and gum arabic were from Sigma Chemical Co. (St. Louis, MO).

Seed germination. Seeds were soaked in concentrated sulfuric acid for 45 min, rinsed twice with sterile deionized water, dusted with Phaltan and Benomyl, and incubated in the dark between moist germination papers at 28°C. The ages of seedlings are expressed relative to the first appearance of an emerging radicle, which generally occurred after 2 to 3 wk of incubation. Elongating epicotyls did not become green.

Determination of seedling moisture and neutral lipid contents. Moisture content was determined by measuring the loss in mass upon drying grated nuts at 105°C for 24 h. Neutral lipid content was determined by Soxhlet extraction of grated seeds with hexane for 18 h followed by determination of the loss in mass.

Homogenization and subcellular fractionation. Seeds or the seed portion of seedlings (5 g), were homogenized in 50 mL of cold Grinding Medium (GM) (0.6 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 20 mM dithiothreitol, 50 mM Tris-Cl buffer, pH 7.5) using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was centrifuged at $10,000 \times g$ for 30 min at 4°C. Three layers resulted: a thin floating lipid layer (the fat pad), a liquid layer (the fat-free supernatant), and a pellet of solid material. The fat pad was suspended in 50 mL of cold GM and washed by centrifugation ($10,000 \times g$, 30 min, 4°C). This produced a fat pad that was resuspended in 50 mL of cold GM and extracted three times with 50 mL of diethyl ether. After the third extraction, the turbid region at the interface between the aqueous and organic layers was recovered with a pipette. Remaining traces of ether were removed from it with a stream of nitrogen, and the material was resuspended in 10 mL of cold GM. This fraction is identified as the "ether-extracted fat pad."

Determination of enzyme activity. Lipase activity was measured by real-time titration of the fatty acids released from a substrate emulsion consisting of 18% (wt/vol) triacylglycerol stabilized with 4.2% (wt/vol) gum arabic (16,17). The reaction vessel was jacketed with a housing connected to a circulating controlled-temperature water bath, which held the reaction temperature constant at 25°C. The set-point pH of the titrating pH meter was varied as necessary to measure the dependence of lipase activity on pH. Enzyme activity was expressed in units of katals (kat), one katal corresponding to the release of one mole of fatty acids per second.

To determine thermal stability, 2 mL aliquots of enzyme solution were incubated for 30 min in 0.1 M Tris-Cl buffer, pH 8.5, at various temperatures, and residual activity was determined at 25°C and pH 8.5.

The effect of pH on enzyme stability was measured at pH 8.5 after dilution of samples of total homogenate with 2 vol of 0.1 M citrate-phosphate (pH 4–7), Tris-Cl (pH 8), or glycine buffers (pH 9, 10) and incubation for 30 min at 25°C. The resulting activities are expressed relative to that of total homogenate held at pH 8.5, after correction for the dilution by buffer.

The effects of detergents on enzymatic activity were determined by adding these to the GM before homogenization, incubating the homogenate for 10 min at room temperature once it was made, and determining the residual lipase activities. These are expressed relative to the activity of a homogenate prepared in detergent-free GM.

Partial purification of lipases. All procedures were performed at 4°C. Seedlings (14 g; 2 wk old) were homogenized in 110 mL of GM containing 1 mM taurocholic acid (THA), 1 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.05% Tween-20. The supernatant (90 mL) produced by sequential centrifugation at $10,000 \times g$ (30 min) and $100,000 \times g$ (1 h) was adjusted to 80% saturation (4°C) with ammonium sulfate and incubated overnight. The pellet obtained by centrifugation ($8,000 \times g$, 20 min) was dissolved in 30 mL of 10 mM Tris-Cl, pH 8.5 and dialyzed overnight against 4 L of this buffer. The dialysate was applied to a 40 × 2.5 cm column of DEAE-Sephadex A-50, which was then washed with four column volumes of fresh dialysis buffer followed by a linear gradient of NaCl from 0 to 0.5 M in dialysis buffer (1.2 L). Fractions (15 mL) were collected and assayed for protein and lipase activity. Protein eluting during column chromatography was detected by measuring the absorbance at 280 nm. Protein concentrations were subsequently quantitated by the method of Bradford (18), using the Bio-Rad protein assay kit in the "micro" assay format, and bovine gamma globulin as the protein standard (Bio-Rad Laboratories, Richmond, CA).

All data represent the averages of at least two determinations. Replicates agreed within 5%.

RESULTS AND DISCUSSION

Germination rate and neutral lipid content. Typical germination percentages for nuts from the current crop year were 15–20%, in agreement with previous reports of poor retention of viability by this species during storage (10,11). *Umbellularia californica* seeds are rich in neutral lipid: dormant nuts contained 74% (mass basis) hexane-extractable material. The neutral lipid content declined upon germination, reaching 43% 5 wk after germination (Fig. 1). The moisture con-

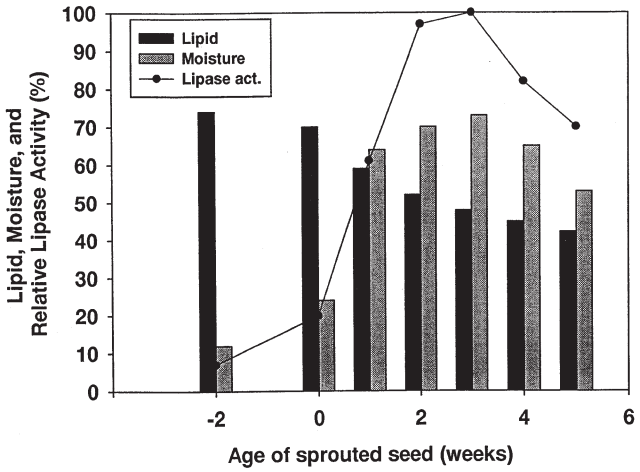

FIG. 1. Lipase activity (crude homogenates), neutral lipid, and moisture levels (%, weight basis) in *Umbellularia californica* seeds upon germination. Maximal lipase activity: 0.67 μkat/g dry wt, using olive oil as substrate. Data at –2 weeks of age represent the condition of dormant seeds.

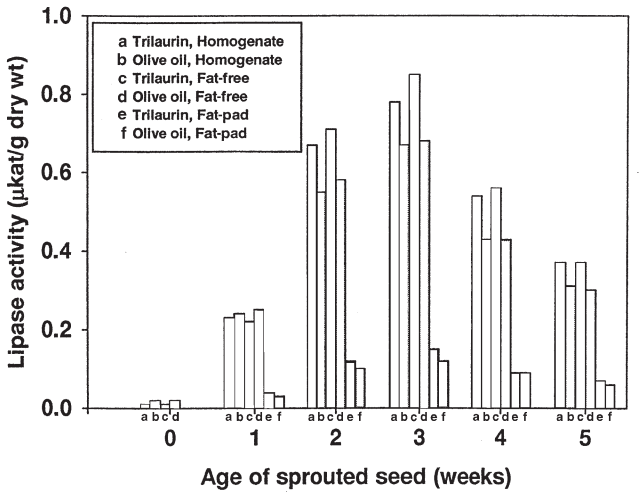

FIG. 2. Changes in lipase activities (total homogenate, fat-free homogenate, and ether-extracted fat pad) upon germination.

tent rose from about 12% in the dormant state to a high of 72% 3 wk after germination and then fell with further incubation (Fig. 1).

Lipase activity. Lipase activity, measured against olive oil (which contains primarily oleic acid, C18:1n-9), was barely detectable in dormant nuts. Significant activity appeared upon germination, increasing to a peak value of 0.6 to 0.7 μkat/g dry wt, about 20-fold above the lipase activity level of dormant seeds, in the second and third weeks following visible germination (Fig. 1). Thereafter, lipase levels declined.

When seedling homogenates were subjected to simple fractionation by centrifugation, followed by ether extraction of the fat pad layer, lipolytic activity was detected primarily in the fat-free layer, with small amounts in the ether-extracted fat pad (Fig. 2). No activity was detected in the particulate fraction. This indicates that the lipase is not primarily associated with the lipid body membranes, which partition into the fat pad layer under the conditions used here (1). Lipase activity was located mainly in the fat-free portion of homogenates throughout the first 5 wk of germination, with no significant appearance of activity in the lipid body fraction (Fig. 2).

Despite the fact that lauric acid is the predominant fatty acid in the storage lipids of *U. californica* seeds, the crude lipolytic activity of germinating seeds did not show hydrolytic specificity for this fatty acid: throughout germination, trilaurin and olive oil (which contains primarily triolein) were hydrolyzed at comparable rates by both total homogenates and subcellular fractions (Fig. 2).

In an effort to maximize the sensitivity of lipase detection, the abilities of the detergents THA (1 mM), Tween-20 (0.05%), and CHAPS (1 mM) to increase apparent lipase activity were investigated. Although these additions stimulated activity, none achieved a substantial increase (Table 1). This is consistent with the suggestion (above) that the lipase is not predominantly bound to lipid body membranes, since detergent treatment of a membrane-bound enzyme might be expected to induce a greater stimulation of activity than seen here. The greatest activity increase was achieved by the joint

TABLE 1
Effects of Detergents on the Activity of the *Umbellularia californica* Seedling Lipase Activity[a]

	Relative activity					
	Substrate: trilaurin			Substrate: olive oil		
Detergent	Homogenate	Fat-free	Fat pad[b]	Homogenate	Fat-free	Fat-pad[b]
None	100 (0.78[c])	100 (0.85)	100 (0.15)	100 (0.67)	100 (0.68)	100 (0.12)
1 mM THA[d]	135	139	120	132	140	136
1 mM CHAPS[d]	147	155	150	160	166	160
0.05% Tween-20	159	168	140	155	162	150
1 mM THA + 1 mM CHAPS + 0.05% Tween	165	178	153	162	168	157

[a]Agents were added to Grinding Medium prior to homogenization of 2-wk-old seedlings.
[b]Ether extracted.
[c]Actual lipolytic activities, in μkat/g dry weight of seedling.
[d]THA, taurocholic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate.

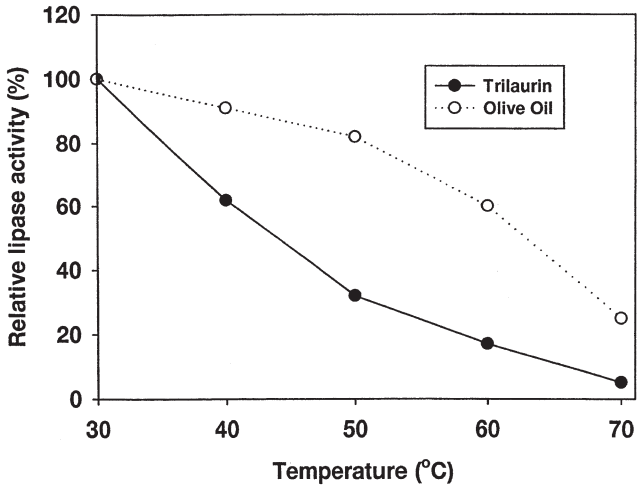

FIG. 3. Temperature stability of the lipolytic activity in crude homogenates of 2-wk-old seedlings. Following a 30-min incubation at the indicated temperatures, residual activity was measured against trilaurin and olive oil by titrimetric assay at pH 8.5, 25°C. Activities are expressed relative to those of a sample of crude homogenate held on ice until assay: against trilaurin: 0.60 μkat/g dry wt; against olive oil: 0.50 μkat/g dry wt.

addition of all three detergents, but even in this case the increase was less than 80% (Table 1).

pH optima and temperature sensitivity of the lipolytic activity. Using total homogenates of 2-wk-old seedlings, the optimal pH for the hydrolysis of all triacylglycerols examined (tributyrin, trilaurin, and olive oil) was 8.5. The activity maximum was not a broad peak: half-maximal activities for all three substrates investigated occurred at pH values of *ca.* 7.75 and 9.25.

The lipolytic activity in total homogenates was generally unaffected by a 30-min incubation at pH values between 6 and 9, but suffered partial inactivation outside this pH region, where lipolytic activity toward olive oil fell off as much as twice as fast as that toward trilaurin. This is consistent with the possible presence of multiple lipases with different substrate ranges and physical properties. The activity was heat-sensitive, with significant losses seen even during 30-min incubations at temperatures as low as 40°C (Fig. 3). The thermal decay curve of the activity toward trilaurin differed substantially from that toward olive oil, in line with the postulate of multiple lipases.

Substrate specificity of the lipolytic activity. A limited test of the substrate range of the lipolytic activity in homogenates of 2-wk-old seedlings validated the impression that the crude *U. californica* lipolytic activity does not exhibit selectivity for lauric acid. Total homogenate and the fat pad fraction (ether-extracted) derived from it showed highest lipolytic activity toward tristearin, and substantial activities toward all other lipids tested (Table 2). The fat-free fraction, which contained the bulk of the activity in the homogenate, exhibited its highest activity toward trilaurin, but also showed substantial activity toward all other substrates examined (Table 2). These results are in general agreement with those of Hellyer *et al.* (15), who employed a heterotriacylglycerol substrate to determine that homogenates of *U. californica* seedlings were lipolytically active toward acylglycerol esters of saturated fatty acids from C8 to C18 in length. However, these authors (15) reported that the lipolytic activity toward trilaurin was threefold greater than that toward unsaturated C18 acylglycerols, whereas our data show a much smaller difference between these activities (Table 2). These apparent discrepancies may be due to the use by Hellyer *et al.* (15) of a mixed acylglycerol substrate, whereas the data presented here were determined with homoacylglycerols.

Partial purification. Essentially all the lipase activity, and 23% of the protein, in a 100,000 × *g* supernatant of total ho-

TABLE 2
Substrate Specificity of the Lipolytic Activity of Crude Homogenates of *Umbellularia californica* (2-wk-old seedlings)

	Lipase activity (μkat/g dry wt)				
Preparation	Tributyrin	Trilaurin	Tripalmitin	Tristearin	Olive oil
Total homogenate	0.45	0.79	0.84	1.00	0.67
Fat-free fraction	0.40	0.85	0.81	0.80	0.68
Fat-pad fraction	0.13	0.14	0.14	0.15	0.13

TABLE 3
Partial Purification of *Umbellularia californica* Lipases

		Activity (μkat)		Specific activity (nkat/mg)		Purification (fold)	
Step	Protein (mg)	TL	Olive oil	TL	Olive oil	TL	Olive oil
Supernatant (10,000 × *g*)	3494.7	2.5	2.0	0.72	0.57	1.0	1.0
Supernatant (100,000 × *g*)	3376.0	2.5	2.0	0.74	0.59	1.0	1.0
80% $(NH_4)_2SO_4$ precipitate	793.5	2.4	1.9	3.0	2.4	4.2	4.2
DEAE-Sephadex:							
Lipase A	97.0	1.3	0.68	13.0	7.0	18.1	12.3
Lipase B	160.0	1.0	0.95	6.3	5.9	8.8	10.4

[a]TL, trilaurin.

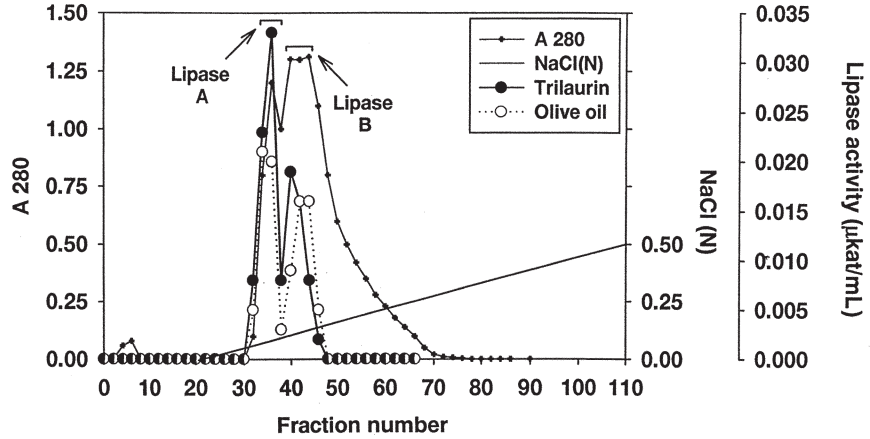

FIG. 4. DEAE-Sephadex A-50 chromatography of the lipolytic activity from 2-wk-old seedlings. Protein, measured as absorbance at 280 nm: (+), lipase activity with substrate trilaurin (●) and olive oil (○). Fractions 32–37 were pooled and designated lipase A. Fractions 39–46 were pooled and designated lipase B.

mogenate was precipitated by addition of ammonium sulfate to 80% saturation (Table 3). Chromatography of the redissolved ammonium sulfate pellet on DEAE-Sephadex A-50 resulted in the partial separation of two peaks of lipolytic activity (Fig. 4). One of these (lipase A, fractions 32–37) eluted at a NaCl concentration of about 65 mM. The other (lipase B, fractions 39–46) eluted at a NaCl concentration of about 100 mM. Degrees of purification of between 8- and 20-fold were achieved for these two activities (Table 3). It is notable that the specific activity of lipase A toward trilaurin was roughly twofold greater than that toward olive oil, whereas lipase B displayed similar specific activities toward both substrates (Table 3). It is possible that lipase A displays substantial selectivity for trilaurin, a feature that would become apparent with further purification.

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