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Fatty Acid-Elongating Activity in Rapidly Expanding Leek Epidermis[1]

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A microsomal fatty acid elongase activity measured in epidermis of rapidly expanding leek (*Allium porrum* L.) was 10-fold higher in specific activity than preparations from store-bought leek. These preparations elongated acyl chains effectively using endogenous or supplied primers. Elongation of C20:0 was specifically inhibited by 2 μM cerulenin, and labeling experiments with [^{3}H]cerulenin labeled two polypeptides (65 and 88 kD). ATP was required for maximal elongase activity in expanding leaves but was lost in nonexpanding tissues. Both [^{14}C]stearoyl-coenzyme A (CoA) and [^{14}C]stearate were maximally elongated in the presence of ATP. Addition of fully reduced CoA, however, inhibited [^{14}C]stearate elongation, suggesting that stearoyl-CoA synthesis was not a prerequisite for elongation. Furthermore, microsomes preincubated with [^{14}C]stearoyl-CoA plus ATP resulted in loss of radiolabel from the acyl-CoA pool without a corresponding loss in elongating activity. The lack of correlation between elongating activity and the label retained in the putative acyl-CoA substrate pool suggests that acyl-CoAs may not be the immediate precursors for elongation and that ATP plays a critical, yet undefined, role in the elongation process. We propose that an ATP-dependent elongating activity may generate the long-chain fatty acids required for wax biosynthesis.

The outer aerial surfaces of a plant are protected from the environment (i.e. drought, pathogens, insects, UV light) by long-chain, hydrophobic wax components derived from saturated fatty acids (Gulz, 1994). In vitro, the saturated fatty acids (of 18 or more carbons in length) are elongated in membrane fractions of epidermal cells (Kolattukudy and Buckner, 1972; Cassagne and Lessire, 1978). This elongation process (referred to as elongase activity) involves a similar series of reactions to those required for de novo fatty acid synthesis, i.e. condensation of a primer with malonyl-CoA, reduction, dehydration, and a second reduction, to produce a fatty acid chain two carbons longer than the starting primer (Fehling and Mukherjee, 1991). In mammalian and yeast systems, fatty acids are synthesized by large, multifunctional enzymes. However, in plant and bacterial fatty acid synthases, these activities are contained on discrete polypeptides. The recent isolations of both a jojoba elongase-condensing enzyme clone encoding a 60-kD protein (Lassner et al., 1994) and the Arabidopsis *FAE1* condensing enzyme clone (James et al., 1995) provide evidence that plant elongases are also multisubunit enzymes.

The regulation of fatty acid elongation in plants, the specific substrates and cofactors involved in the elongation process, and the number of elongases catalyzing very-long-chain fatty acid biosynthesis are not well understood. Although an epidermis-specific elongase has been partially purified from leek (*Allium porrum* L.), with an enrichment of four polypeptides between 50 and 65 kD (Bessoule et al., 1989), characterization of elongases in general has been slow, because of low enzyme activities, enzyme instability in detergents (Podack et al., 1974; Lessire et al., 1985; Murphy and Mukherjee, 1989; Créach and Lessire, 1993), and the problem of isolating multiple elongases from multiple membrane locations (Cassagne et al., 1987; Imai et al., 1994). Therefore, we have examined rapidly expanding leek epidermis as a source of high specific activity elongase.

We found that microsomes isolated from epidermal tissue of rapidly expanding leeks were approximately 10-fold higher in elongase specific activity than epidermis from store-purchased leeks. This activity was comparable to activities of membrane-bound elongases associated with triacylglycerol biosynthesis, which correlates with in vivo rates of erucic acid biosynthesis (J. Jaworski, personal communication). We used these leek microsomes to reexamine specific properties of the epidermal elongase, particularly with respect to cofactor requirements and substrate specificities. In addition, we labeled two putative condensing enzymes from a solubilized elongase fraction with [^{3}H]cerulenin.

MATERIALS AND METHODS

Chemicals

[^{3}H]Cerulenin (94 GBq mmol^{-1}) was a gift from Dr. Penny von Wettstein-Knowles (Carlsberg Laboratory, Copenhagen, Denmark). [2-^{14}C]Malonyl-CoA (2.2 GBq mmol^{-1}) was synthesized according to the method of Rutkoski and Jaworski (1978). [^{14}C]Stearic acid (2.2 GBq mmol^{-1}) was purchased from Amersham. Triton X-100 (Surfact-AMPs X-100) was from Pierce. Protein assay re-

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Abbreviations: C*x*:*y*, fatty acid notation where *x* = number of carbon atoms and *y* = number of double bonds; C18:0, stearate; CoASH, fully reduced CoA; KAS; 3-ketoacyl-acyl carrier protein synthase; PC, phosphatidylcholine; Z 3–10, *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1propanesulfonate.

agent, Coomassie brilliant blue R-250, and silicic acid (Biosil A; 200–400 mesh) were from Bio-Rad. Boron trichloride (in 10% methanol) was from Alltech Associates, Inc. (Deerfield, IL). All other chemicals were from Sigma. Soybean PC (Sigma) was L-α-PC type II-S. Acyl-CoA synthetase (Sigma) was from *Pseudomonas*.

[^{14}C]Stearoyl-CoA (2.2 GBq mmol^{-1}) was synthesized according to the method of Taylor et al. (1990) with the following modifications. [^{14}C]Stearic acid (10 mM; 22 GBq) was dried under N_2 and dissolved in 100 μL of 95% ethanol. Ammonium hydroxide (1–2 drops) was added, and the solution was heated at 55°C for 2 min and dried again under N_2. The residue was redissolved in 1% Triton X-100 (100 μL) and added to a reaction that contained the following components: 0.1 M Mops-NaOH, pH 7.5, 20 mM $NaHCO_3$, 1 mM DTT, 5 mM CoASH, 10 mM ATP, 10 mM $MgCl_2$, and 0.15 unit of acyl-CoA synthetase in a final volume of 1 mL. The reaction proceeded for 2 h at 35 to 37°C with constant stirring; a second aliquot of enzyme (0.15 unit) was added and the reaction continued for another 2 h. The reaction was stopped with HCl to give a final concentration of 0.1 M. The reaction mixture was extracted once with an equal volume of chloroform:methanol (2:1, v/v); the lower phase was discarded and the upper aqueous phase was extracted with an equal volume of hexane. The hexane phase was discarded, and the lower aqueous phase was extracted twice with an equal volume of water-saturated butanol. The butanol phase was collected, concentrated under N_2 to 0.5 mL, and applied to a small column of silicic acid (0.2 g), equilibrated with butanol. Chloroform (1 mL) was then applied to the column to remove lipid. The [^{14}C]stearoyl-CoA was eluted with 1 mL of methanol, dried under N_2, and redissolved in 0.1% Triton X-100 acidified to pH 2.5.

Plant Material

Leek (*Allium porrum* L.) leaves were removed from store-purchased mature leeks with a razor blade, leaving 1 cm of leaf above each bulb. The roots were trimmed to 1 cm, and the white bulbs were planted in Metro-mix 200 (Grace-Sierra, Milipitas, CA). Leaves were regrown over 10 d (23°C, 16-h photoperiod). Epidermis was peeled from 10-d-old leek leaves, frozen in liquid N_2, and stored at −80°C until used.

Preparation of Membrane Fractions and Detergent Solubilization

Frozen leek epidermis (usually 6–12 g) was pulverized in liquid N_2 and further homogenized with a Polytron in 80 mM Hepes-KOH, pH 7.2, 2 mM EDTA, 320 mM Suc, 2 mM DTT, and 0.3 mM PMSF (three 1-s bursts at half-maximum speed). The homogenate was filtered through three layers of Miracloth (Calbiochem) and centrifuged at 10,000*g* for 20 min (HB6 rotor). The supernatant was collected and centrifuged at 106,000*g* for 1 h (Sorvall TH 641 or AH 629 rotor), and the resulting microsomal pellet was resuspended in 80 mM Hepes-KOH, pH 7.2, 1 mM DTT, and 15% glycerol. Microsomes were homogenized briefly with a 2-mL Dounce homogenizer and either used immediately or frozen in liquid N_2 and stored at −70°C. The protein content was determined by the method of Bradford (1976), with a commercial protein assay reagent and BSA as the standard.

Elongase activity was solubilized from membranes at a protein concentration of 2 mg mL^{-1} with 10 mM Hepes-KOH, pH 7.2, 1 mM DTT, 10% glycerol, and varying concentrations of Z 3–10 to give detergent to protein ratios of 0:1 to 25:1. The microsomal suspensions were incubated at 4°C for 1 h with shaking. In control experiments, the suspensions were shaken under identical conditions without detergent. The soluble fraction was recovered after centrifugation at 106,000*g* for 1 h in a Sorvall T 1270 rotor; the pelleted membranes were resuspended in the same solubilization buffer to determine residual activity. In initial tests to determine the effect of various detergents, 0.1% detergent was added to the elongase assays. When a phospholipid dispersion was included in the assay, it was prepared by sonicating the lipid in 80 mM Hepes-KOH, pH 7.2, 1 mM DTT, followed by centrifugation as described by Monroy et al. (1973).

Molecular Weight Determination

A portion of the Z 3–10-solubilized leek preparation (0.2 mL) was filtered through a 0.22-μm filter and applied to a Superose 6 HR 10/30 fast protein liquid chromatography column (Pharmacia) that was pre-equilibrated with 80 mM Hepes-KOH, pH 7.2, 1 mM EDTA, 10% glycerol, 1 mM DTT, and 20 mM Z 3–10. Fractions (0.5 mL) were collected at a flow rate of 0.25 mL/min and assayed for elongase activity. Molecular weights were estimated from plots of K_{av} as a function of log M_r.

Elongase Assays

Leek microsomes (20 μg of protein) or solubilized protein fractions (7–15 μg) were assayed for 30 min at 30°C (25-μL assays) in 80 mM Hepes-KOH, pH 7.2, 5% glycerol, 1 mM DTT, 0.5 mM NADPH, 1 mM ATP, 5 mM $MgCl_2$, 100 μM [2-^{14}C]malonyl-CoA, and in the presence or absence of 15 μM acyl-CoA (C18:0-, C20:0-, or C22:0-CoA in a final concentration of 0.005% Triton X-100). In some experiments, a range of stearoyl-CoA and stearate concentrations (5–160 μM in 0.17% Triton X-100) was tested for elongation in the presence of 100 μM [^{14}C]malonyl-CoA. In other experiments, [^{14}C]stearoyl-CoA and [^{14}C]stearate were examined at concentrations of 10 to 15 μM in the presence of 100 μM unlabeled malonyl-CoA. Elongase assays were stopped with 50 μL of 4 N KOH in 80% methanol. In a blank reaction, the methanol-KOH was added before the enzyme. The lipids were saponified for 1 h at 80°C and acidified to <pH 3.0 with cold 6 N HCl. The fatty acids were then extracted twice with 400 μL of cold hexane. The pooled hexane fractions were dried under N_2 in scintillation vials and counted in 6 mL of Ultima-Gold scintillation cocktail (Packard Instrument Co., Meriden, CT) or derivatized for product analyses as described below.

Product Analyses

The products of the elongase reaction were methylated in a sealed tube with 0.5 mL of boron trichloride in methanol (10% w/v) at 60°C for 15 min. Water (2.5 mL) was added, and the fatty acid methyl-esters were extracted two times with 3 mL of hexane. The fatty acid methyl-esters were concentrated under N_2 and separated on KC_{18} reverse-phase TLC plates (Whatman), developed in acetonitrile:tetrahydrofuran (85:15, v/v). For the analysis of lipid products, the elongase assays were stopped with 2 reaction volumes of butanol:acetic acid (5:2, v/v). Two reaction volumes of H_2O were then added, and the phases were separated by centrifugation. The upper butanol phase was removed, and the lower phase was re-extracted with 2 reaction volumes of butanol. The butanol phases were pooled; the volume was reduced under a stream of N_2, and the lipid products were separated on Kieselgel 60 F-254s (Merck) high-performance TLC plates (10 × 20 cm), developed in butanol:acetic acid:water (5:2:3, v/v/v). TLC products were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Inhibition of Elongase Activity by Cerulenin

The effect of cerulenin on elongase activity in leek microsomes was determined by adding to the assay the indicated concentrations of cerulenin (0–100 μM in 0.5% ethanol) or by preincubation of microsomes for 10 min with cerulenin, followed by addition of substrates (30-min assay).

[^{3}H]Cerulenin was also used to label cerulenin-binding proteins. Leek microsomes (100 μg in 100 μL volume) were labeled for 30 min at 30°C with 3 μM [^{3}H]cerulenin (94 KBq) in 80 mM Hepes-KOH, pH 7.2, and 5% glycerol and in the presence or absence of 0.5 mM DTT. In some experiments, microsomes were labeled for 10 min with [^{3}H]cerulenin and then incubated for 20 min longer in the presence of 200 μM unlabeled cerulenin. Microsomes were washed twice with 500 μL of buffer (minus cerulenin) and pelleted for analysis by SDS-PAGE. To determine the native molecular mass of [^{3}H]cerulenin-binding proteins, 1 mg of leek microsomes was labeled with 3 μM [^{3}H]cerulenin in 80 mM Hepes-KOH, pH 7.2, 5% glycerol, and 0.5 mM DTT at 30°C for 30 min. Unlabeled cerulenin was added to give a final concentration of 200 μM, and the incubation continued for 10 min. Microsomes were pelleted at 106,000*g* for 1 h, and the pellet was solubilized in 10 mM Hepes-KOH, pH 7.2, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, and Z 3–10 at a detergent to protein ratio of 4.5 (3 mg mL^{-1} protein). The detergent-extracted microsomes were again pelleted at 106,000*g* for 1 h, and 200 μL of the supernatant were applied to a Superose 6 gel filtration column as described above. Fractions (0.5 mL) were collected and 25 μL of each fraction were counted for ^{3}H label. The fractions containing the highest radioactivity were precipitated with 12.5% TCA in an ice-salt bath for >30 min. The precipitated protein was recovered by centrifugation at 18,000*g* for 30 min at 4°C. Depending on the enzyme preparation, between 2000 and 6500 dpm of ^{3}H-labeled protein were precipitated in the peak elongase activity fraction. All pellets were resuspended in 10 μL of SDS-PAGE sample buffer (Schagger and von Jagow, 1987). Samples were boiled for 5 min and separated on 10% SDS-PAGE minigels (Bio-Rad) at 100 V. The radioactive gels were stained for 1 h with 0.025% Coomassie brilliant blue R-250 in 40% methanol, 7% acetic acid and destained completely with 50% methanol, 10% acetic acid. Subsequently, the gels were dehydrated (with shaking) for 10 min each in 25% acetic acid, 50% acetic acid, and glacial acetic acid and then agitated for 1 h in approximately 5 gel volumes of En^{3}Hance (Dupont). Gels were then placed in an excess of cold water and agitated for 30 min. The gels were dried and exposed to preflashed (Bonner and Laskey, 1974) Kodak X-Omat AR film for 7 to 21 d at −70°C.

RESULTS AND DISCUSSION

Preparation of High Specific Activity Elongase from Leek Epidermis

The purification of membrane-bound elongases from plant and animal sources has proven to be a difficult achievement (Podack et al., 1974; Bolton and Harwood, 1977; Bernart and Sprecher, 1979; Lessire et al., 1985; Bessoule et al., 1989; Fehling et al., 1992). This is primarily due to low specific activities, enzyme instability, and detergent inhibition of component elongase activities. To isolate fatty acid biosynthetic enzymes of high specific activity in plants, Roughan and Nishida (1990) emphasized the need for controlled growth environments and the use of rapidly expanding tissues. We have used their recommendations in our study of elongases.

Rapidly expanding leek leaves were readily obtained by regrowing trimmed store-purchased leeks. Epidermal microsomes from 10-d-regrown leeks were prepared by a modification of previously reported protocols for store-purchased leeks (Lessire et al., 1985) or etiolated leek seedlings (Schneider et al., 1993) and yielded about 0.2 mg of protein per g of epidermis. The elongase activity was stable to freezing at −70°C, with less than 10% activity lost upon thawing. Typically, the elongase specific activity in microsomes prepared from rapidly expanding leek epidermis was 10-fold higher (21 nmol h^{-1} mg^{-1}) than activity from store-purchased leek epidermis (2.0 nmol h^{-1} mg^{-1}) when assayed in the presence of ATP and stearoyl-CoA (Fig. 1A). An ATP-independent elongase had a similar specific activity in regrown or store-purchased leeks (2.8 nmol h^{-1} mg^{-1}). Elongase activities in our store-purchased leeks were also comparable to activities previously reported (Agrawal et al., 1984; Agrawal and Stumpf, 1985). In Figure 1B, the products of elongase activity (assayed as [^{14}C]malonyl-CoA incorporation in the presence or absence of ATP) are shown. Microsome preparations typically elongated fatty acids to C26:0 or longer. Note that, in addition to an overall stimulation of elongation in the presence of ATP, short-chain products (<C20:0) were also synthesized. Calculations of specific activities were therefore based on elongated products only, unless otherwise noted.

A comparison of the elongation rates in 10-d rapidly expanding leeks and store-purchased leeks indicated that

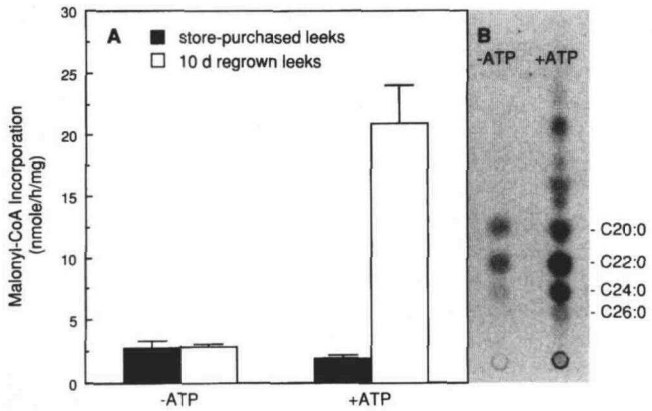

Figure 1. A, Total [^{14}C]malonyl-CoA incorporation into hexane-extractable products. Epidermal microsomes were prepared from store-purchased leeks or 10-d-regrown leeks. Assays were performed in the presence of 15 μM stearoyl-CoA and in the presence or absence of 1 mM ATP. Each column is the mean and each bar is the ±SE for elongase activity in three different microsome preparations. B, Elongation products in regenerated leek (assayed with or without ATP with 15 μM stearoyl-CoA) separated by reverse-phase TLC.

the ATP-dependent elongase activity increased in the rapidly expanding leek epidermis. Therefore, this activity may be critical to the normal synthesis of wax precursors during regrowth of leaves. Under conditions in which the leek leaf was no longer expanding, the ATP-dependent activity was completely lost. Conversely, the ATP-independent elongase had a similar specific activity in both tissues.

Substrate Specificity of the Leek Epidermal Elongase

In leek microsomes, [^{14}C]malonyl-CoA incorporation into elongated product was linear up to 40 min in the presence or absence of stearoyl-CoA. Stearoyl-CoA was typically used in assays at a final concentration of 15 μM, since micelles form at higher acyl-CoA concentrations (Powell et al., 1981). However, the effect of a range of stearoyl-CoA and stearate concentrations (5–160 μM in 0.17% Triton) was also tested (Fig. 2). [^{14}C]Malonyl-CoA incorporation increased from 11.0 nmol h^{-1} mg^{-1} (0 μM stearoyl-CoA) to 25.0 nmol h^{-1} mg^{-1} with 160 μM stearoyl-CoA. Incorporation of [^{14}C]malonyl-CoA was greater, however, in the presence of unlabeled stearate (when stearate concentrations were >15 μM) (Fig. 2). In a standard elongase assay (with ATP plus 15 μM unlabeled stearoyl-CoA or stearate), both long-chain fatty acids and short-chain products were detected (as in Fig. 1B). This was due to the incorporation of [^{14}C]malonyl-CoA into unknown endogenous primers as well as incorporation into the added acyl primer.

It is widely accepted that acyl-CoAs are the in vivo substrates for the membrane-localized elongases in plants (Agrawal and Stumpf, 1985; Lessire et al., 1989). In experiments in which stearate is the supplied primer, it has been suggested that stearate is activated to stearoyl-CoA, since acyl-CoA synthetase activity is present in microsomes (Ichihara et al., 1993). This enzyme uses the cofactors ATP and CoASH to catalyze the activation of fatty acids to acyl-CoAs (Lessire and Cassagne, 1979). Since the safflower acyl-CoA synthetase has a K_m of 24 μM for CoASH (Ichihara et al., 1993), stimulation of elongase activity by CoASH was expected if CoASH is involved in the synthesis of an acyl-CoA substrate. Therefore, we compared [^{14}C]stearoyl-CoA elongation with [^{14}C] stearate elongation (as opposed to total [^{14}C]malonyl-CoA incorporation in Fig. 2) and examined the effect of CoASH on the elongation of [^{14}C]stearate (Fig. 3). [^{14}C]Stearate elongation decreased with increasing concentrations of CoASH (25–800 μM). This contrasts with the results of Cassagne and Lessire (1978), who showed that activity increased with the addition of 0.5 mM CoASH plus 2 mM ATP. In that study, however, the effect of ATP on elongase activity (in the absence of CoASH) was not examined. Because acyl-CoA synthesis from [^{14}C]stearate could occur if endogenous CoASH was present in microsomes, CoASH was depleted according to Agrawal and Stumpf (1985) in rapidly expanding leek microsomes. No significant difference in [^{14}C]malonyl-CoA incorporation into hexane-extractable counts (17 nmol h^{-1} mg^{-1}) was observed, however, between CoA-depleted or nondepleted microsomes. Furthermore, supplied CoASH had no effect on [^{14}C]malonyl-CoA incorporation (data not shown).

In previous studies, the results with CoASH were inconsistent. CoASH stimulated [^{14}C]malonyl-CoA incorporation by 10% in leek microsomes (Agrawal et al., 1984), but in germinating pea microsomes, CoASH inhibited incorporation (Bolton and Harwood, 1977). CoASH also inhibited malonyl-CoA incorporation in rat liver and brain microsomes, possibly because of competition with malonyl-CoA (Cook, 1982; Reichelt et al., 1994). However, when rat brain microsomes were washed to remove free CoA, free fatty acids were still elongated as long as ATP was present (Cook, 1982). Taken together, these experiments suggest

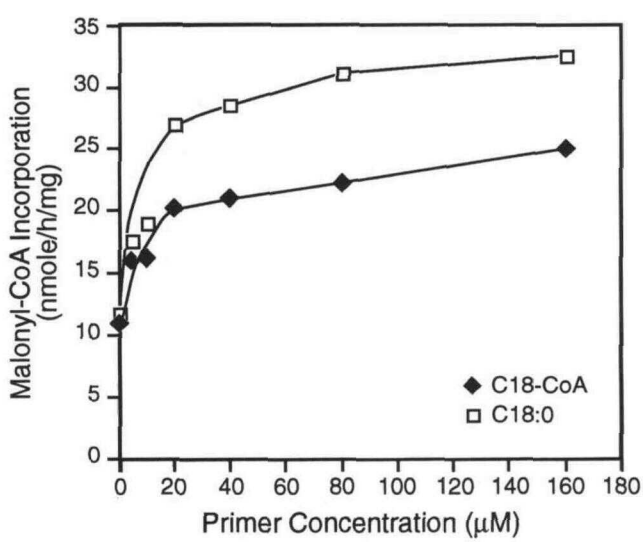

Figure 2. Effect of stearate and stearoyl-CoA concentration on [^{14}C]malonyl-CoA incorporation into hexane-extractable counts in leek microsomes. Stearate (NH_4^+ salt) and stearoyl-CoA were sonicated and added to the assay in a final Triton concentration of 0.17%. [^{14}C]Malonyl-CoA (100 μM) was the labeled substrate.

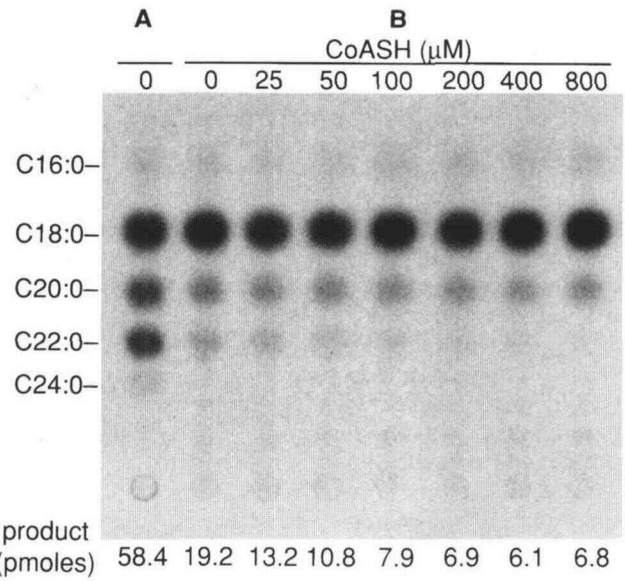

Figure 3. Reverse-phase analysis of TLC products of ATP-dependent elongation in leek microsomes. Products with 15 μM [^{14}C]stearoyl-CoA (A) or 15 μM [^{14}C]stearate (NH_4^+ salt; B) as the labeled precursor. CoASH (0–800 μM) was added to elongase assays in the presence of [^{14}C]stearate. The concentration of total products formed per reaction (>C18:0) is listed below each lane.

that acyl-CoAs may not be a required intermediate for elongation.

ATP Requirement for Maximal Elongase Activity

The ATP requirement for maximal elongation of fatty acid and fatty acyl-CoA substrates is of interest, since the biochemistry of a two-carbon addition from malonyl-CoA (i.e. condensation, reduction, dehydration, and reduction) does not have an obvious requirement for ATP. In mammalian tissues the ATP requirement for fatty acid elongation differs: ATP is essential for maximal elongase activity in rat brain mitochondria and microsomes (Cook, 1982; Aeberhard and Menkes, 1986; Christiansen et al., 1986), whereas in rat liver ATP is not required (Bernart and Sprecher, 1977). In plants Cassagne and Lessire (1978) reported that ATP increases the [^{14}C]stearoyl-CoA elongation rates 2-fold in leek microsomes. This increased elongase activity was attributed to an acyl-CoA synthetase, which resynthesizes stearoyl-CoA from free stearate and CoASH, the two products generated by contaminating microsomal acyl-CoA thioesterases (Abdul-Karim et al., 1980). However, as our study has shown, the ATP requirement (by the regrown leek elongase) cannot be explained by acyl-CoA synthesis from free fatty acids (either supplied or generated by the hydrolysis of supplied acyl-CoAs).

Stymne and Glad (1981) demonstrated that in microsomes of developing soybean cotyledons, acyl exchange readily occurs between acyl-CoAs and PC. Therefore, we preincubated leek microsomes with [^{14}C]stearoyl-CoA in the absence or presence of ATP to determine the fate of the acyl moiety (Fig. 4) and to determine whether subsequent elongation rates were affected (Fig. 5). Preincubation of microsomes with [^{14}C]stearoyl-CoA in the absence of ATP (Fig. 4A) resulted in a 97% loss of ^{14}C label from the acyl-CoA pool within 2 min; the major labeled product was PC, with moderate levels of label in phosphatidylethanolamine and neutral lipids (free fatty acids and triacylglycerol). If microsomes were preincubated with [^{14}C]stearoyl-CoA plus ATP, the ^{14}C-labeled acyl-CoA pool was also depleted but only by 50% in 15 min (Fig. 4B).

The effect of [^{14}C]stearoyl-CoA preincubation on subsequent elongase activity is shown in Figure 5. Despite a complete loss in the [^{14}C]stearoyl-CoA pool following a 15-min preincubation (minus ATP), subsequent ATP-dependent elongase activity was reduced only 55% compared to microsomes preincubated with buffer (Fig. 5, cf. lanes 3 and 5). The ATP-independent elongase activity was reduced 100% compared to the 15-min preincubation with buffer (Fig. 5, cf. lanes 4 and 6). In contrast, preincubation with [^{14}C]stearoyl-CoA plus ATP resulted in no loss of elongating activity compared to a standard 30-min assay without preincubation (data not shown). Since there was no correlation between the loss of radioactivity from the [^{14}C]stearoyl-CoA pool and subsequent elongating activity, the results further support our view that stimulation of elongase activity by ATP is unrelated to the synthesis of stearoyl-CoA. It is possible that ATP is involved in binding the substrate to the enzyme. However, two other possibilities are that ATP serves as an energy source to form an intermediate substrate or is required for the production of an adenylated intermediate.

Solubilization of Elongase Activity

Triton X-100 was previously used to solubilize the ATP-independent elongase activity from membrane fractions of

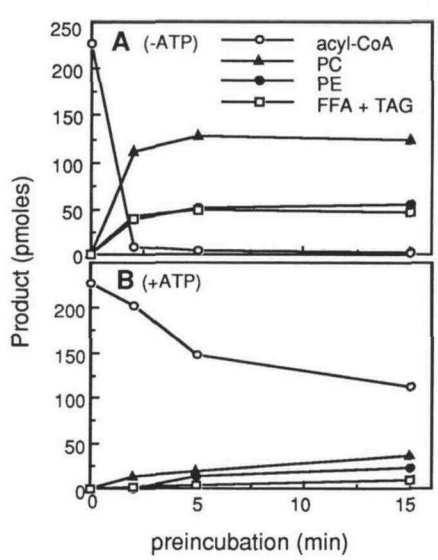

Figure 4. Time course of preincubation of microsomes with 10 μM [^{14}C]stearoyl-CoA only (A) or with 10 μM [^{14}C]stearoyl-CoA plus ATP (B). Reactions were separated on silica gels (butanol:acetic acid: water; 5:2:3, v/v/v). The concentration of radiolabeled product was quantified from the lipid bands by phosphorimaging. PE, Phosphatidylethanolamine; FFA, free fatty acid; TAG, triacylglycerol.

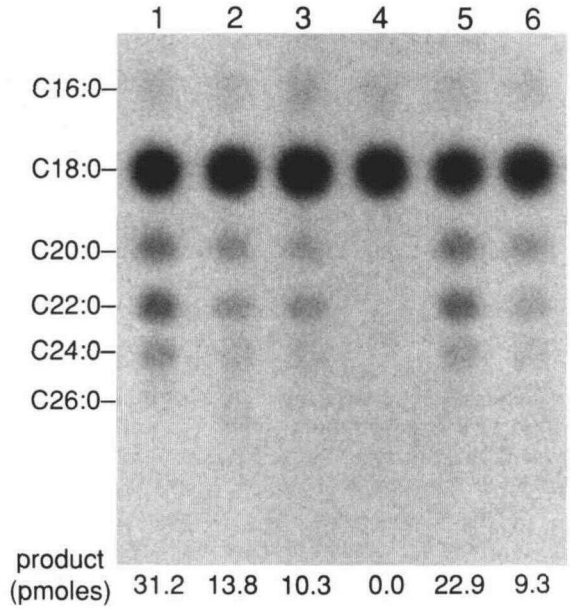

Figure 5. Reverse-phase TLC analysis of ATP-dependent and -independent elongation products in leek microsomes with 10 μM [^{14}C]stearoyl-CoA. Lane 1, Products of the standard elongase assay (30 min) plus ATP. Lane 2, Products of the standard elongase assay minus ATP. Lane 3, Preincubation (15 min) with [^{14}C]stearoyl-CoA, followed by elongase assay plus ATP. Lane 4, Preincubation (15 min) with [^{14}C]stearoyl-CoA, followed by elongase assay minus ATP. Lane 5, Preincubation (15 min) with buffer, followed by elongase assay plus ATP. Lane 6, Preincubation (15 min) with buffer, followed by elongase assay minus ATP. The amount of product formed (>C18:0) is listed below each lane.

leek epidermis and developing seeds (Agrawal and Stumpf, 1985; Lessire et al., 1985; Murphy and Mukherjee, 1989; Créach and Lessire, 1993). Triton, however, produces large micelles and absorbs UV light, thereby interfering with the monitoring of protein absorbance. Leek elongase activity (measured as [^{14}C]malonyl-CoA incorporation) was therefore compared in the presence of several zwitterionic and nonionic detergents (Table I) to determine whether a specific detergent preserved elongase activity. In the presence of 0.1% detergent, the highest activity was obtained with Z 3–10. However, all detergents resulted in some inhibition of the ATP-dependent elongase activity from regenerated leek.

Leek microsomes were mixed with Z 3–10 at several detergent to protein ratios (0:1–25:1, w/w) to optimize solubilization of elongase activity. Optimal solubilization occurred at a detergent to protein ratio of approximately 4% (2 mg mL^{-1} protein): 55% of the microsomal protein, but only 25% of the elongase activity was recovered in the soluble fraction (assayed with ATP and stearoyl-CoA). Because inhibition of elongase activity occurred at all Z 3–10 concentrations, further work will be necessary to stabilize this enzyme in the presence of detergent. Phospholipids are often added to stabilize and stimulate membrane-bound enzymes (Sloan et al., 1987). However, attempts to reactivate the Z 3–10-solublized (or nonsolublized) elongase activity with a crude soybean PC fraction were not successful; all concentrations of soybean PC between 0.1 and 3.0 mg mL^{-1} either had no effect or were inhibitory. In contrast, Bessoule et al. (1989) previously reported a slight activation of a Triton X-100-solubilized elongase activity in the presence of soybean PC. That activity, however, was the ATP-independent elongase and was approximately 30-fold lower in specific activity compared to our Z 3–10-solubilized enzyme.

Two criteria used to estimate solubilization of a membrane-bound elongase were (a) that the solubilized elongase activity remained in the supernatant following a 100,000*g* centrifugation and (b) that it eluted after the void volume on a gel filtration column. The Z 3–10-solubilized ATP-dependent elongase activity eluted from a Superose 6 column in two peaks; the major peak (18% of total activity applied) eluted with a molecular weight of 220,000 ± 17,000 and a minor peak (7% of total activity applied) eluted with a molecular weight of 29,500 ± 3,200 (Fig. 6). Approximately 75% of the total elongase activity applied to the column was lost, which further indicated that this enzyme was very unstable. Similar activity peaks were also obtained if microsomes were solubilized and eluted by gel filtration in the presence of 0.25% Triton X-100 (K.J. Evenson and D. Post-Beittenmiller, unpublished data).

Effect of Cerulenin on Elongase Activity

KAS is a condensing enzyme for de novo fatty acid synthesis and is comparable to elongase-condensing enzyme activity. There are also multiple KAS isozymes in plants (Shimakata and Stumpf, 1982). KAS I activity is irreversibly inhibited by cerulenin in microorganisms and plants (D'Agnolo et al., 1973; Roberts and Leadlay, 1983; Siggaard-Andersen, 1988, 1990). In contrast, cerulenin is a reversible inhibitor of the KAS II isozyme (Shimakata and Stumpf, 1982) and reversibly inhibits the synthesis of some polyketide-derived products (Martin and McDaniel, 1975; Omura, 1981). Cerulenin also inhibits ATP-independent elongation beyond C20:0 in leek microsomes (Agrawal et

Table I. *Effect of various detergents (0.1%) on [^{14}C]malonyl-CoA incorporation into hexane-extractable counts in leek microsomes*

Each value is the mean elongase activity from two different microsome preparations, assayed in the presence of ATP and stearoyl-CoA.

Detergent	Specific Activity	Percent Control (no detergent)
	nmol h^{-1} mg^{-1}	
Control	20.5	100
Z 3–10	13.8	67.2
N-Octyl α-D-gluco pyranoside	13.2	64.4
Triton X-100	12.1	59.2
3-[(3-Cholamidopropyl)dimethylammonio]-7-propanesulfonate	12.0	58.6
N-Dodecyl-*N*,*N*-dimethy-3-ammonio-7-propanesulfonate	8.5	41.4
Polyoxyethylene 8 lauryl ether	4.9	24.1
n-Dodecyl α-D-maloside	2.0	9.8

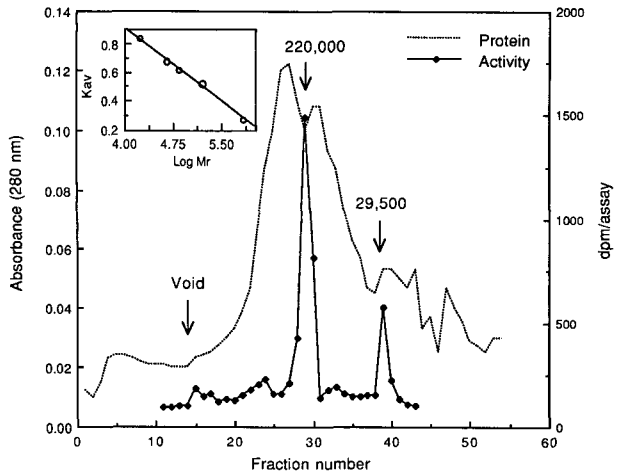

Figure 6. Fast protein liquid chromatography Superose 6 elution profile of ATP-dependent elongase activity. Fractions (0.5 mL) were collected and 15-μL aliquots were assayed. Molecular weight standards were: bovine thyroglobulin, 670,000; bovine γ-globulin, 158,000; chicken ovalbumin, 44,000; horse myoglobulin, 17,000. The elution profile of the two activity peaks was consistently observed in four solubilized preparations.

al., 1984; Schneider et al., 1993). We therefore examined the effect of cerulenin on ATP-dependent elongation (Fig. 7). Leek elongase activity (assayed with stearoyl-CoA) was inhibited approximately 50% by 2 μM cerulenin. The inhibition was biphasic, indicating that two different activities may be inhibited. The 50% inhibition values are also significantly lower than values previously reported for cerulenin inhibition of elongation (40 and 150 μM) (Agrawal et al., 1984; Schneider et al., 1993). Furthermore, inhibition of an elongase activity from developing *Lunaria* seeds was observed only at high cerulenin concentrations (50% inhibition at 120 μM) (Fehling et al., 1992), and cerulenin does not inhibit *Brasssica napus* seed elongase (A. Hlousek-Radjocic, personal communication).

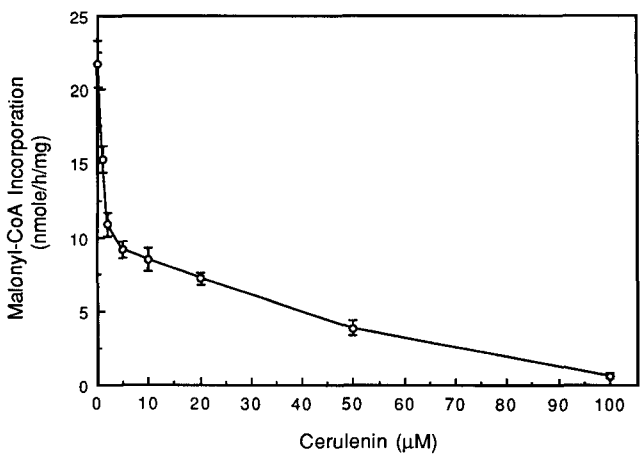

Figure 7. Effect of cerulenin on [^{14}C]malonyl-CoA incorporation into hexane-extractable counts. Assays (30 min) included 15 μM stearoyl-CoA plus ATP. Results are the means ± SE of four independent determinations.

Inhibition of leek elongase activity by cerulenin was further examined in the presence of three acyl-CoA primers (Fig. 8) to determine whether an increased inhibition of specific elongation steps (>C18:0) occurred. With C18:0-CoA as a primer, the major product was C22:0, which made up 51% of total product. In the presence of 10 μM cerulenin, however, formation of C22:0 product was inhibited 87% and the amount of C20:0 doubled. With C20:0-CoA as the added primer, the major product was also C22:0 (45% of total product), but formation of this product was inhibited 84% in the presence of 10 μM cerulenin. In contrast, if C22:0-CoA was the added primer, product formation beyond C22:0 was not greatly inhibited in the presence of cerulenin. These results confirm and extend the findings of Agrawal et al. (1984) regarding a cerulenin-sensitive elongase in leek. A key point revealed by our study was that the specific inhibition of the C20:0-elongation step was significantly greater than the overall inhibition (measured as [^{14}C]malonyl-CoA incorporation).

To determine whether a cerulenin-sensitive elongase could be preferentially solubilized, leek microsomes were treated with Z 3–10 at a detergent to protein ratio of 4 (Table II). Synthesis of C22:0 product was strongly inhibited by cerulenin in both the Z 3–10-soluble and -insoluble fractions, similarly to control fractions incubated with cerulenin. Therefore, a cerulenin-sensitive elongase was not preferentially solubilized. The C20:0-to-C22:0 elongation step was also inhibited by the detergent Z 3–10, since the amount of C22:0 product (Table II) was less than the sum of the soluble plus pellet fractions (when compared to the control pellet). Compare the second elongation step to the elongation of C18:0 to C20:0, which was not inhibited by Z 3–10: the amount of C20:0 product in the soluble plus pellet fractions (28.7 pmol) was greater than in the control pellet.

The low 50% inhibition value for cerulenin (for total malonyl-CoA incorporation into hexane-extractable counts) and the decreased accumulation of C22:0 product

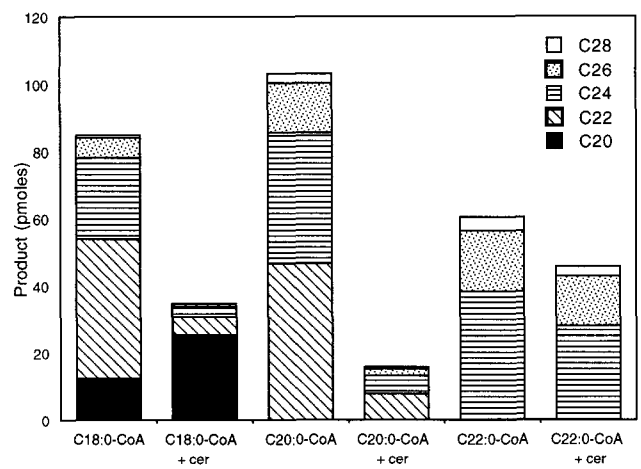

Figure 8. Effect of 10 μM cerulenin (cer) on [^{14}C]malonyl-CoA incorporation into elongated products. Microsomes were assayed in the presence of various acyl-CoA primers. Products were separated by reverse-phase TLC and the concentration of elongated product was quantified by phosphorimaging.

Table II. *Cerulenin inhibition of Z 3–10-solubilized leek elongase activity*

Microsomes were solubilized at a detergent to protein ratio of 4 (2 mg mL^{-1}) and centrifuged at 106,000*g*. The supernatant and resuspended pellet were assayed for elongase activity in the presence or absence of 10 μM cerulenin. Assays (30 min) included 15 μM stearoyl-CoA, 1 mM ATP, and 100 μM [^{14}C]malonyl-CoA as the labeled substrate. Reaction products were separated by reverse-phase TLC and products (>C18:0) were quantified by phosphorimaging.

Treatment	Supernatant			Pellet				
	C20:0	C22:0	C24:0	C20:0	C22:0	C24:0	C26:0	C28:0
				product (pmol)				
Control (no Z 3–10)								
−Cerulenin	0.3	0	0	24.8	55.8	26.7	3.6	1.0
+Cerulenin (10 μM)	0	–	–	38.6	4.1	1.8	0.5	0
Z 3–10 solubilized								
−Cerulenin	16.6	3.9	0	12.1	3.0	0	–	–
+Cerulenin (10 μM)	14.3	0.2	0	11.9	0	–	–	–

indicated that cerulenin was a potent inhibitor of the C20:0-elongating activity. To determine whether this inhibition was irreversible, microsomes were preincubated for 10 min with 10 μM cerulenin and then diluted to 2 μM cerulenin and assayed for elongase activity. [^{14}C]Malonyl-CoA incorporation was inhibited 62%, similar to reactions in which microsomes were both preincubated and assayed for elongase activity in the presence of 10 μM cerulenin (data not shown). These results suggested that cerulenin irreversibly inhibited elongase activity. Therefore, [^{3}H]cerulenin was tested as a possible radioactive tag for an elongase-condensing enzyme. Leek microsomes were labeled for 30 min with 3 μM [^{3}H]cerulenin in either the presence or absence of DTT (to determine whether DTT nonspecifically reacted with cerulenin). In other experiments, microsomes were labeled for 10 min with [^{3}H]cerulenin, followed by a 20-min chase with 200 μM unlabeled cerulenin to minimize nonspecific labeling. In each case, three polypeptides of 56, 65, and 88 kD were clearly labeled when analyzed by SDS-PAGE and fluorography (Fig. 9A). Leek microsomes were also labeled with [^{3}H]cerulenin, solubilized with Z 3–10, and applied to a Superose 6 gel filtration column. The solubilized and gel filtration fractions were counted for radioactivity, and two ^{3}H-labeled polypeptides of 65 and 88 kD (Fig. 9B) were detected by fluorography of SDS-PAGE gels. These radiolabeled polypeptides eluted at an apparent molecular mass of 220 kD and correlated with elution of the ATP-dependent elongating activity (Fig. 6). No ^{3}H-labeled polypeptides were associated with the 29.5-kD gel filtration peak shown in Figure 6.

The subunit masses of the ^{3}H-labeled polypeptides in this study differed with the [^{3}H]cerulenin-labeled KAS I polypeptides (45–46 kD) in barley chloroplasts (Siggaard-Andersen, 1990), suggesting that membrane-bound condensing enzymes are larger than the comparable soluble condensing enzymes of fatty acid synthase. The most strongly labeled polypeptide shown in Figure 9B (65 kD) was similar in subunit mass to a leek polypeptide previously enriched by partial purification of an ATP-independent elongase (Bessoule et al., 1989). In developing *B. napus* seeds, a molecular mass of 60 kD was also reported for the elongase-condensing enzyme (Imai et al., 1994). DNA clones encoding 56- to 60-kD elongase-condensing enzymes have also been isolated from jojoba and Arabidopsis (Lassner et al., 1994; James et al., 1995). Further purification is necessary to determine whether a leek elongase-condensing activity positively correlates with a [^{3}H]cerulenin-tagged protein and whether epidermal and seed elongases share similarities in structure and function.

CONCLUSIONS

In this paper we have described an ATP-dependent fatty acid-elongating activity of significantly higher specific activity than previously reported. We propose that this elongase generates the long-chain fatty acids required for wax biosynthesis in rapidly expanding tissues. With the increased elongating activity, it may now be possible to correlate maximal elongation rates in vitro with in vivo

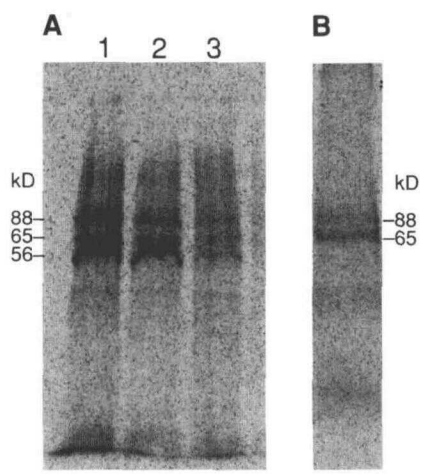

Figure 9. A, SDS-PAGE (10% gel) and fluorography of [^{3}H]cerulenin-labeled leek microsomes (100 μg of protein labeled per lane). *Lane 1, Microsomes labeled for* 30 min in the presence of 0.5 mM DTT; lane 2, microsomes labeled for 30 min in the absence of DTT; lane 3, microsomes labeled for 10 min (minus DTT) followed by a 20-min chase with 200 μM cold cerulenin. B, SDS-PAGE and fluorography of [^{3}H]cerulenin-labeled solubilized elongase activity (which eluted at a molecular weight of 200,000 by gel filtration). Standards were visualized by Coomassie blue staining.

rates of wax deposition and ultimately to determine the regulatory steps in wax biosynthesis.

Because of the number of steps catalyzed by epidermal and seed elongases and the large number of putative elongases uncovered through genetic (von Wettstein-Knowles, 1982), inhibitor (Mikkelsen, 1978), and substrate-specificity studies (Agrawal and Stumpf, 1985), it is apparent that the isolation and characterization of elongases will reveal differences in enzyme function and regulation. The differential sensitivity to cerulenin by monocot and dicot elongases and the potential use of [^{3}H]cerulenin as an elongase-condensing enzyme tag in protein expression studies may help elucidate both the elongation process and the intracellular location of wax biosynthetic enzymes.

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