Properties of Lysophosphatidylcholine Acyltransferase from *Brassica napus* Cultures

Tara L. Furukawa-Stoffer, Riley M. Boyle, Amber L. Thomson, Magdalena A. Sarna, and Randall J. Weselake*

Department of Chemistry and Biochemistry, University of Lethbridge, Lethbridge, Alberta, T1K 3M4 Canada

ABSTRACT: Acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT; EC 2.3.1.23) catalyzes the acyl-CoA-dependent acylation of lysophosphatidylcholine (LPC) to produce PC and CoA. LPCAT activity may affect the incorporation of fatty acyl moieties at the *sn*-2 position of PC where PUFA are formed and may indirectly influence seed TAG composition. LPCAT activity in microsomes prepared from microspore-derived cell suspension cultures of oilseed rape (*Brassica napus* L. cv Jet Neuf) was assayed using [1-^{14}C]acyl-CoA as the fatty acyl donor. LPCAT activity was optimal at neutral pH and 35°C, and was inhibited by 50% at a BSA concentration of 3 mg mL^{-1}. At acyl-CoA concentrations above 20 μM, LPCAT activity was more specific for oleoyl (18:1)-CoA than stearoyl (18:0)- and palmitoyl (16:0)-CoA. Lauroyl (12:0)-CoA, however, was not an effective acyl donor. LPC species containing 12:0, 16:0, 18:0, or 18:1 as the fatty acyl moiety all served as effective acyl acceptors for LPCAT, although 12:0-LPC was somewhat less effective as a substrate at lower concentrations. The failure of LPCAT to catalyze the incorporation of a 12:0 moiety from acyl-CoA into PC is consistent with the tendency of acyltransferases to discriminate against incorporation of this fatty acyl moiety at the *sn*-2 position of TAG from the seed oil of transgenic *B. napus* expressing a medium-chain thioesterase.

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Elucidation of the mechanisms of lipid biosynthesis in oleaginous crops provides a valuable foundation for developing metabolic engineering strategies to alter the FA composition of seed oil. In developing oilseeds, the biosynthesis of membrane phospholipids is closely linked to TAG biosynthesis and involves reactions catalyzed by an assortment of membrane-bound acyltransferases (1–4). The specificity properties of the acyltransferases can influence the types and relative amounts of FA moieties that are ultimately incorporated into TAG. In the *sn*-glycerol-3-phosphate pathway leading to TAG, acyl-CoA:lysophosphatidate acyltransferase (LPAAT; EC 2.3.1.51) catalyzes the acylation of lysophosphatidate to generate phosphatidate and CoA (1,4). Studies with microsomes from developing seeds of *Brassica napus* have indicated that the resident LPAAT discriminates against unusual FA moieties including erucoyl (22:1)- (5,6) and lauroyl (12:0)-CoA (7). The decreased specificity of endogenous LPAAT for 12:0-CoA was believed to be the reason for decreased incorporation of 12:0 at the *sn*-2 position of TAG from *B. napus* genetically engineered to express a medium-chain thioesterase from *Umbellularia californica* (8,9). Increased incorporation of 12:0 at the *sn*-2 position of *B. napus*, however, was achieved through coexpression of the medium-chain thioesterase from *U. californica* and a 12:0-CoA-preferring LPAAT from *Cocos nucifera* (10).

There are other possible routes for the incorporation of FA moieties at the *sn*-2 position of TAG. Membrane-bound acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT; EC 2.3.1.23) catalyzes the acyl-CoA-dependent acylation of lysophosphatidylcholine (LPC) to produce PC and CoA (1,11). In developing seeds of oleaginous crops such as *B. napus*, LPCAT activity may affect the incorporation of FA at the *sn*-2 position of PC where PUFA are formed (1). *sn*-1,2-DAG, derived from PC *via* the reverse reaction of CDP-choline:1,2-DAG cholinephosphotransferase (EC 2.7.8.2), may be used as substrate in the acyl-CoA-dependent biosynthesis of TAG (12–15). PC can also serve as a source of *sn*-2 FA moieties in the acyl-CoA-independent biosynthesis of TAG *via* donation of the FA moiety to *sn*-1,2-DAG (16). The resulting LPC product could then serve as substrate for LPCAT. As well, in studies with developing safflower (*Carthamus tinctorius*) seed, there is evidence for acyl-exchange between the FA at the *sn*-2 position of PC and the acyl-CoA pool (1,17–20). Phospholipase A_2, which catalyzes the hydrolysis of the *sn*-2 FA from PC, may also have a role in generating LPC (21,22) for utilization by LPCAT. Thus, the activity level and the specificity properties of LPCAT may influence the FA composition of seed oil. Studies on microsomal LPCAT have been restricted mainly, however, to developing safflower seeds (1,23,24). Bernerth and Frentzen (6) reported that *B. napus* LPCAT was capable of discriminating against 22:1-CoA, but no data were presented. A deeper understanding of the role of oilseed LPCAT in storage lipid biosynthesis could be realized through the availability of molecular probes, but there have been no reports on the purification of the plant enzyme to homogeneity or the identification of an encoding cDNA. Given the global importance of *B. napus*, the current study has focused on examining the properties of microsomal LPCAT from microspore-derived cell suspension cultures of this oilseed crop. Until now, this enzyme

*To whom correspondence should be addressed at Dept. of Chemistry and Biochemistry, University of Lethbridge, 4401 University Dr., Lethbridge, Alberta T1K 3M4, Canada
E-mail: weselake@uleth.ca

Abbreviations: 12:0, lauroyl; 16:0, palmitoyl; 18:0, stearoyl; 18:1, oleoyl; 22:1, erucoyl; ACBP, acyl-CoA binding protein; LPAAT, acyl-CoA:lysophosphatidate acyltransferase; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase.

activity has been essentially uncharacterized in *B. napus*. In addition to providing fundamental knowledge on the properties of *B. napus* LPCAT, our characterization studies suggest that, along with LPAAT specificity, LPCAT specificity also may represent a barrier to the incorporation of medium-chain FA at the *sn*-2 position of TAG.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Lauric acid (59 Ci mol^{-1}), [1-^{14}C]oleic acid (56 Ci mol^{-1}), and [1-^{14}C]stearic acid (54 Ci mol^{-1}) were obtained from Amersham Biosciences Inc. (Brie d'Urfé, Québec, Canada). [1-^{14}C]Palmitic acid (56 Ci mol^{-1}) was from NEN Life Science Products, Inc. (Boston, MA). Acyl-CoA were synthesized from radiolabeled FA using acyl-CoA synthetase (25). Merck silica gel 60H, used for preparing TLC plates, was from VWR Canlab (Mississauga, Ontario, Canada). Ecolite^{TM} (+) biodegradable scintillant was from ICN Biomedicals, Inc. (Irvine, CA). Dye reagent concentrate for protein assays was from Bio-Rad (Hercules, CA). HPLC-grade solvents were from BDH, Inc. (Toronto, Ontario, Canada). All other lipids and biochemicals were of the highest purity available and were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Cell culture and preparation of microsomes. The microspore-derived cell suspension culture of *B. napus* L. cv Jet Neuf was maintained essentially according to Orr *et al.* (26). The culture was grown in 125-mL Erlenmeyer flasks on a rotary shaker (150 rpm) at 25°C under constant light with an intensity of about 30 μmol m^{-2} s^{-1}. One-third of the mass of cells was routinely transferred to fresh medium at 2-wk intervals. The remainder of the cells, obtained after 2 wk of culture, was washed with water over a nylon sieve and blotted with filter paper to remove excess water; the fresh weight was then determined. Cells were ground in 4 vol of grinding buffer (10 mM MOPS-NaOH, pH 7.2, 0.4 M sorbitol, 0.5 mM EDTA) using a chilled mortar and pestle. The homogenate was centrifuged at 3000 × *g* for 20 min at 4°C and the resulting pellet discarded. The supernatant was filtered through glass wool to remove the lipid layer and centrifuged at 20,000 × *g* for 30 min at 4°C. The resulting pellet was discarded, and the supernatant was filtered through glass wool. The supernatant was then centrifuged at 100,000 × *g* for 1 h at 4°C, and the resulting pellet was resuspended in a volume of grinding buffer equivalent to 1/20 of the original mass of cells. The resuspended microsomes were divided into 100-μL aliquots, which were frozen with liquid N_2 and stored at –80°C.

Enzyme assays. The assay for LPCAT activity was adapted from previously described methods (24,27). Assays were conducted in a volume of 0.7 mL in 10-mL glass tubes at 30°C. Unless indicated otherwise, the standard reaction mixture consisted of 80 mM Tris-HCl, pH 7.5, 0.21 M sorbitol, 0.13 mM EDTA, 100 μM [1-^{14}C]oleoyl-CoA (0.5 Ci mol^{-1}), 75 μM 18:1-LPC, and about 25 μg of microsomal protein. Acyl-CoA was used to initiate the enzyme reaction, which was usually allowed to proceed for 10 min. The reaction was terminated by the addition of 3 mL chloroform/methanol (2:1, vol/vol). For zero reaction time controls, the solvent mixture was added to the reaction mixture before addition of microsomes. Phase separation was induced by the addition of 1.1 mL 0.9% (wt/vol) KCl. Following brief centrifugation, the upper aqueous phase was removed, and 1.5 mL of the lower organic phase was transferred to a 3-mL glass tube. The solvent was dried under a gentle stream of N_2 gas, and the sides of the glass were rinsed with a small volume of chloroform/methanol (2:1, vol/vol). Once completely dried, the samples were resuspended in 70 μL chloroform/methanol (2:1, vol/vol), and 50 μL was applied in 1.5-cm lanes to a 20 × 20 cm TLC plate coated with 0.5 mm silica gel 60H. Seventy microliters of PC solution (10 mg mL^{-1} in chloroform) was applied on top of the dried lipid extract to act as a carrier. A control lane with only PC was used to establish the migration distance of the phospholipid. The TLC plate was developed with one ascent of chloroform/methanol/NH_4OH (33:45:5, by vol). Based on the PC control, visualized with iodine vapor, corresponding radioactive PC spots in the adjacent lanes containing the reaction mixtures were scraped from the plate and radioactivity was determined in 5 mL Ecolite^{TM}(+) scintillant. A phosphoimaging system (Canberra-Packard Canada Ltd., Mississauga, Ontario, Canada) was used in preliminary experiments to confirm that radiolabeled PC was resolved from other lipid classes. The protein content of the microsomes was determined using the Bio-Rad protein microassay based on the Bradford method (28), using BSA as a standard.

RESULTS AND DISCUSSION

Effect of various parameters on LPCAT activity. The time course for production of PC, catalyzed by microsomal LPCAT under the buffer conditions of the standard assay, is depicted in Figure 1A. Production of PC was linear for up to about 20 min. To stay within the linear range of the time course, subsequent enzyme reactions for further characterization work were limited to 10 min. When assayed at the same temperature, the time course for production of PC catalyzed by solubilized microsomes containing LPCAT activity from developing safflower seed was shown previously to be linear for about 30 min (23). The effect of increasing protein content in the LPCAT reaction mixture is shown in Figure 1B. Enzyme activity was directly proportional to the microsomal protein content up to about 70 μg. Based on these results, the standard assay was routinely performed with about 25 μg of protein in the reaction mixture. The dependence of microsomal LPCAT activity on pH is shown in Figure 1C. Enzyme activity was maximal at pH 7.0. The neutral pH optimum is consistent with an earlier report on the effect of pH on microsomal LPCAT activity from developing safflower seeds (24). The effect of reaction temperature on microsomal LPCAT activity is shown in Figure 1D. Under the conditions of the assay, the enzyme exhibited an apparent temperature optimum of 35°C. Assays conducted for a longer period of time may affect enzyme stability and subsequently the apparent temperature optimum. Stymne and Stobart (20)

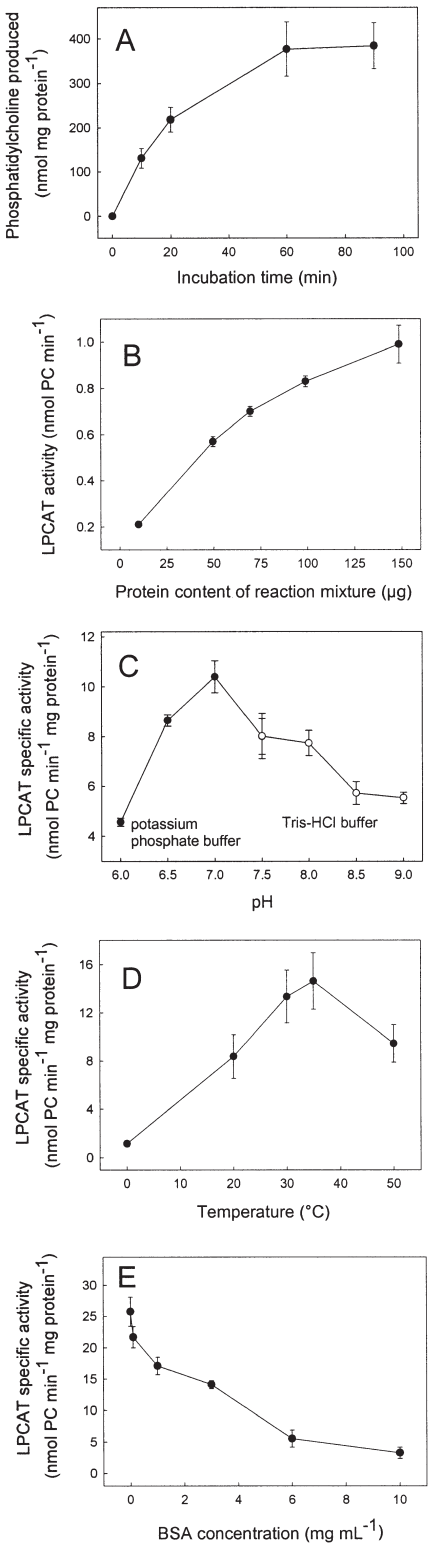

FIG. 1. Effect of various reaction conditions on microsomal acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) activity. (A) Time course for the production of PC; (B) effect of the protein content of the reaction mixture; (C) effect of pH; the total concentration of buffer species was 80 mM; (D) effect of reaction temperature; (E) effect of BSA concentration. For (B) to (E), enzyme-catalyzed reactions were allowed to proceed for 10 min. Each data point represents the average of three determinations ± SEM.

observed increasing LPCAT activity up to 35°C in 1.5-min reactions conducted with safflower microsomes. The effects of higher temperatures on the rate of the enzyme-catalyzed reaction, however, were not evaluated in their investigations.

The effect of various concentrations of BSA on microsomal LPCAT activity is depicted in Figure 1E. BSA was inhibitory to microsomal LPCAT activity at all concentrations tested, with 50% inhibition occurring at a BSA concentration of approximately 3 mg mL^{-1}. Early work by Moreau and Stumpf (23), with microsomes from developing safflower seed, indicated that 1 mM BSA inhibited LPCAT activity at a pH value greater than 6. As well, in the presence of BSA, LPCAT exhibited maximum activity at pH 6. In contrast, in the absence of BSA, LPCAT activity was optimal at pH 8. Previous studies with microsomes of developing soybeans (*Glycine max*) (17) and safflower (19) have shown that the inclusion of BSA in the reaction mixture favors acyl-exchange at the *sn*-2 position of PC catalyzed by LPCAT. The reversible binding of acyl-CoA by BSA was presumed to shift the equilibrium toward removal of acyl groups from PC (19). BSA has been shown to have variable effects on the microsomal acyltransferases of storage lipid biosynthesis. For example, acyl-CoA:DAG acyltransferase (EC 2.3.1.20) in particulate fractions from microspore-derived embryos of *B. napus* was stimulated four- to fivefold at a BSA concentration of 3–4 mg mL^{-1} (29). DAG acyltransferase catalyzes the acyl-CoA-dependent acylation of *sn*-1,2-DAG to generate TAG (1). In studies with microsomes from developing safflower seeds, Bafor *et al.* (30) reported that inclusion of BSA in the reaction mixture altered the selectivity of LPAAT for acyl-CoA. Concentrations of BSA greater than 0.25 mg mL^{-1} caused LPAAT to have an increased preference for 18:2-CoA over acyl-CoA containing saturated acyl moieties.

Substrate specificity of LPCAT. The effect of increasing acyl-CoA concentration on microsomal LPCAT activity is shown in Figure 2A. At a concentration of 20 µM acyl-CoA, palmitoyl (16:0)-, stearoyl (18:0)-, and oleoyl (18:1)-CoA were utilized at the same rate by LPCAT. At higher concentrations of acyl-CoA, LPCAT was more active with 18:1-CoA. The enzyme was not active with 12:0-CoA at any of the thioester concentrations tested. The tissue concentration of acyl-CoA in developing seeds of *B. napus* has been determined to be in the range of 3-6 µM (31). With the assumption that the cell suspension cultures exhibit similar acyl-CoA concentrations, *B. napus* LPCAT would be equally effective using 16:0-, 18:0-, or 18:1-CoA in the direction of PC formation. In contrast, LPCAT activity from developing safflower microsomes has been shown to have a much greater specificity for 18:1-CoA in comparison to acyl-CoA composed of saturated FA at thioester concentrations of 20 µM or lower in the reaction mixture (23,24).

The effects of various concentrations (up to 10 µM) of 18:1-LPC on LPCAT activity are depicted in Figure 2B. The effect of higher concentrations of various molecular species of LPC, including 18:1-LPC, is shown in Figure 2C. Utilization rates for 16:0-, 18:0-, and 18:1-LPC were similar at all concentrations tested. From 10–50 µM LPC, 12:0-LPC was a less effective substrate than the other molecular species of LPC. The inability

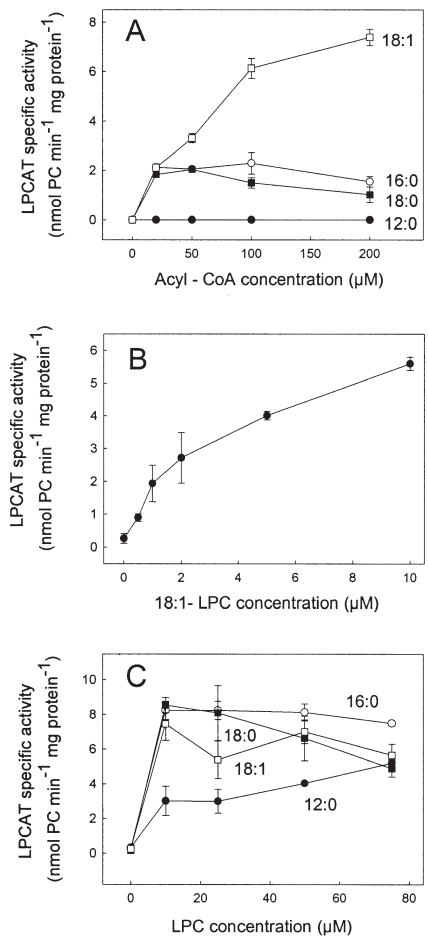

FIG. 2. Effect of substrate concentration on microsomal LPCAT activity. (A) Acyl-CoA specificity; reactions were initiated with [1-^{14}C]acyl-CoA; (B) Effect of 18:1 lysophosphatidylcholine (LPC) at concentrations up to 10 μM; (C) LPC specificity at LPC concentrations up to 75 μM. For B and C, reactions were initiated with [1-^{14}C]18:1-CoA. Reactions were allowed to proceed for 10 min. Each data point represents the average of three determinations ± SEM. For other abbreviation see Figure 1.

of *B. napus* LPCAT to utilize 12:0-CoA was consistent with studies using microsomes from developing safflower seed. Ichihara *et al.* (24) have also found that LPCAT activity in microsomes from developing safflower seeds utilized 16:0-, 18:0-, and 18:1-LPC with essentially equal effectiveness over a range of LPC concentrations. The safflower enzyme, however, utilized 12:0-LPC at a relatively lower rate than LPCAT activity in microsomes from the cell suspension cultures of *B. napus*. It is interesting to note that the ability of *B. napus* and safflower LPCAT activity to utilize 12:0-LPC is different from *B. napus* LPAAT activity from developing seeds, which has been shown to strongly discriminate against 12:0-lysophosphatidate (7).

The role of LPCAT activity in TAG biosynthesis in B. napus. Most research on plant LPCAT has involved assaying the enzyme activity in microsomes. There are a number of uncertainties in working with this heterogeneous assay system. Enzyme activity will depend on factors such as the protein and lipid content of microsomes, the critical micellar concentrations of different molecular species of acyl-CoA, and the presence of acyl-CoA binding proteins (ACBP). Nonetheless, investigations with microsomes have provided a number of insights into the action of LPCAT in developing oilseeds. Considerable research, mainly with developing safflower seed, has suggested that the reverse reaction of LPCAT may facilitate acyl-exchange at the *sn*-2 position of PC with the acyl-CoA pool, thereby creating new opportunities for the incorporation of PUFA into TAG (1). PC with polyunsaturated acyl groups is derived from FA desaturation at the level of PC (1,2,32). Endogenous ACBP may play a role in modulating lipid biosynthesis by altering the availability of acyl-CoA (33,34). Both soluble (33–35) and membrane-bound ACBP (36,37) have been identified and characterized in oilseeds. Soluble recombinant ACBP from *Arabidopsis thaliana* has been shown to protect [^{14}C]oleoyl-CoA against hydrolysis by acyl-CoA hydrolase (EC 3.1.2.14) (34). In the case of LPCAT, ACBP may have a role in reducing the concentration of free acyl-CoA such that acyl exchange is enhanced in similar fashion to the effect of BSA (19). As well, the existence of phospholipid:DAG acyltransferase (EC 2.3.1.158) (16) and phospholipase A_2 (21,22) activity in plants suggests that the forward reaction of LPCAT also could have a physiological role in reacylating LPC in a manner similar to that proposed for mammalian systems (38). Thus, LPCAT activity might participate indirectly in the exclusion of unusual FA from membranes by catalyzing the reacylation of LPC with non-unusual FA.

It has been suggested that the acyl-CoA selectivity properties of endogenous LPAAT activity are the reason for the decreased incorporation of 12:0 at the *sn*-2 position in *B. napus* expressing a medium-chain thioesterase (8). Indeed, earlier specificity studies with microsomal LPAAT from developing seeds of *B. napus* indicated that the enzyme could utilize 12:0-CoA, but with considerably less effectiveness than 18:1-CoA (7). Given the inability of *B. napus* LPCAT to accept 12:0-CoA and the possible reversibility of the cholinephosphotransferase-catalyzed reaction, we suggest that the endogenous LPCAT activity of *B. napus* also may have contributed to the decreased incorporation of 12:0 at the *sn*-2 position of *B. napus* genetically engineered to produce 12:0-CoA. Future metabolic engineering work aimed at further increasing the prevalence of 12:0 or 22:1 at the *sn*-2 position of TAG in seeds of *B. napus* should also take into consideration the activity of LPCAT. Further insights into the role of LPCAT activity in TAG biosynthesis will come from studies with transgenic plants in which the activity of this enzyme is altered through genetic engineering.

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