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LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE FROM IMMATURE COCONUT ENDOSPERM HAVING MEDIUM CHAIN LENGTH SUBSTRATE SPECIFICITY

H. MAELOR DAVIES,* DEBORAH J. HAWKINS and JANET S. NELSEN

Oils Division, Calgene, Inc., 1920 Fifth Street, Davis, CA 95616, U.S.A.

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Key Word Index—*Cocos nucifera*; Palmae; coconut; endosperm; enzymes; membranes; solubilization; lysophosphatidic acid acyltransferase.

Abstract—Immature endosperm of coconut (*Cocos nucifera*) contains a membrane-bound lysophosphatidic acid acyltransferase (LPAAT) having medium chain length substrate specificity appropriate to the biosynthesis of coconut oil. Acyl-CoAs containing 10:0, 12:0 and 14:0 acyl groups are the preferred acyl-donor substrates; acyl-ACPs are not utilized. There is slight preference for 12:0-lysophosphatidic acid (LPA) over 18:1-LPA as acceptor substrate. Treatment of the active membrane fraction with 2.25% (w/v) CHAPS, at a detergent:protein ratio of 48:1 (w/w), in the presence of 1M NaCl solubilized the enzyme in high yield. Solubilization was evidenced by three independent criteria, namely, failure of the activity to sediment at high centrifugal force, behaviour of the activity as a globular protein of apparent M_r 44000 in size-exclusion chromatography, and partial resolution of the activity from many of the membrane proteins on the size-exclusion column. Optimal restoration of LPAAT activity after solubilization required the addition of detergent-treated phospholipids, in addition to a lowering of the detergent and NaCl concentrations.

INTRODUCTION

The lipid reserves in the seeds of many plants comprise triacylglycerols (oil), which are considered to derive from glycerol-3-phosphate and acyl-CoA *via* the Kennedy pathway (reviewed in [1]). The individual, membrane-associated acyltransferase reactions have been assayed *in vitro* and their specificities compared in different species that accumulate triacylglycerols of different acyl compositions (reviewed in [2]). These studies have shown that the acyltransferases responsible for sn-1 and sn-3 acylation are relatively nonspecific with respect to the acyl groups of their substrates and can transfer acyl groups which are not normally found in the reserve triglycerols *in vivo*. However, the acyl-CoA: 1-acyl-sn-glycerol-3-phosphate *O*-acyltransferase (lysophosphatidic acid acyltransferase, EC 2.3.1.51; LPAAT) responsible for sn-2 acylation varies in specificity between species in accordance with the triacylglycerol composition. For example, in rapeseed (*Brassica napus*) embryo membranes, the LPAAT is most active with acyl-donor (acyl-CoA) and acceptor (lysophosphatidate; LPA) substrates containing unsaturated C18 acyl groups, these being the principal groups at sn-2 of the reserve triacylglycerols [3]; very little LPAAT activity is detected when medium-chain (C8–C14) donors and acceptors are tested. In contrast, LPAAT in embryo membranes from species of *Cuphea*, whose seeds accumulate medium-chain triacylglycerols, show considerable acitivity with saturated, medium-chain substrates [4]. Transgenic rapeseed lines have been produced in which the acyl composition of the seed oil has been altered dramatically [5]. For example, up to 50% of the acyl groups have been replaced by medium chains (12:0 and 14:0) [5, 6]. Because of the relative inactivity of rapeseed LPAAT towards such acyl groups [2–4] there is considerable interest in the isolation of specialized LPAATs, so that their genes may be cloned and introduced into the new, transgenic rapeseed varieties in order to increase the proportion of novel fatty acids in their triacylglycerols. Isolation of the LPAAT protein from the membrane environment will also facilitate studies of its mode of action and the basis of its substrate specificity.

There have been several claims of the solubilization of LPAAT from cytoplasmic (microsomal) membranes (for example, [7, 8]) but all appear to have relied upon one indication of apparent solubilization, namely an inability to pellet the activity by high-speed centrifugation. In our experience, this test frequently gives a false-positive result. This may explain why none of the published procedures shows that the supposedly solubilized LPAAT lends itself to protein chromatography in the manner of a typical non-membrane enzyme, and why some have concluded that the LPAAT protein is extremely large or part of a complex [8]. Furthermore, the suitability of the

*Author to whom correspondence should be addressed.

purportedly solubilized LPAAT preparations for purification work has been questioned and it has been suggested that alternative methods will be needed to isolate the gene [9–11]. We have re-examined the solubilization of LPAAT using membrane preparations from immature endosperm of coconut (*Cocos nucifera*). We show that this medium chain-specific enzyme can be solubilized from the membrane, as evidenced by three independent criteria, and that once solubilized it requires phospholipids for activity.

RESULTS AND DISCUSSION

A crude homogenate of endosperm tissue from store-bought coconuts contained medium chain LPAAT activity, as shown by assaying with 12:0-CoA and 12:0-LPA. Activity yields varied considerably between individual coconuts but the highest activities (75–150 pkat mg^{-1} protein) were consistently obtained from the most immature specimens, referred to as 'green', having a whitish brown exocarp. Among these, the best yields were from those having a thin, translucent endosperm. Typically 40% of the activity in the crude homogenate remained after low-speed centrifugation to remove unbroken cells, debris, etc., i.e. in the S1 fraction. After dialysis to lower the NaCl concentration from 3 M to 1 M, and another centrifugation to clarify the dialysate, 30% of the starting homogenate activity remained. High-speed centrifugation divided this activity *ca* equally between supernatant (S2) and pellet (P2) fractions. Further high-speed centrifugation of the S2 fraction failed to sediment additional LPAAT activity.

Considerable evidence has been presented to show that oilseed LPAAT involved in triacylglycerol biosynthesis is associated with cytoplasmic membranes and is specific for acyl-CoA as the donor substrate (reviewed in [2]). Plastidial LPAAT is also considered to be membrane-associated but is active with acyl–acyl carrier protein (ACP), as well as acyl-CoA [2]. In view of the high lipid content of the preparations, we did not attempt to characterize the types of membrane present in the coconut P2 preparation by density gradient centrifugation. But, as this material was completely inactive with 12:0-ACP as donor substrate we considered it equivalent to the other cytoplasmic LPAAT preparations that have been described. The specific activity of this P2 LPAAT preparation was typically 0.8–1.5 pkat μg^{-1} protein. (Activity remaining in the S2 fraction was also inactive with 12:0-ACP as acyl donor and may be associated with cytoplasmic membranes whose sedimentation was compromised during extraction, *e.g.* by association with storage lipid.)

With 12:0-L-α-lysophosphatidate (LPA) as acceptor substrate, the membrane (P2) LPAAT activity varied with the acyl-CoA substrate (Table 1). Relative activities with 10:0-, 12:0- and 14:0- donor substrates varied slightly from preparation to preparation but these were consistently the preferred acyl-CoAs; maximum activity was usually obtained with 12:0-CoA. These results are consistent with the predominance of medium chains and 12:0, in particular, in coconut oil [12] and contrast markedly with the reported specificity of rapeseed microsomal LPAAT [3]. We confirmed, under our extraction and assay conditions, that rapeseed embryo LPAAT shows much less activity with 12:0-CoA than with 18:1-CoA, whether the acceptor substrate is 18:1-LPA or 12:0-LPA (results not shown). This comparison illustrates the specialization of coconut LPAAT in relation to the storage oil composition and provides further evidence that the activity is involved in triacylglycerol biosynthesis.

Table 1. Activities* of LPAAT preparations with 12:0-LPA and various acyl-CoAs

Acyl group of acyl-CoA†	P2 (membrane) activity (fkat)	Reactivated S3 activity (fkat)
6:0	4	1
8:0	35	11
10:0	172	172
12:0	291	300
14:0	174	85
16:0	46	34
18:0	21	12
18:1	105	46

*After subtraction of background activities obtained in absence of exogenously supplied 12:0-LPA. (Without background subtraction the S3 activities were 10–20% higher than those shown, presumably due to the presence of LPAs in the soybean phospholipid preparation.)

†Radiolabelled substrate.

The same acyl-CoA specificity was obtained when assaying coconut LPAAT with 18:1-LPA (results not shown). However, data obtained with 18:1-LPA must be interpreted cautiously, because pre-incubation of the P2 preparation with this substrate for a few seconds resulted in substantial activity loss. (Subsequent assays with 12:0-LPA were then comparably affected.) Measurable activities were obtained only if 18:1-LPA was added to the assay system immediately before starting the reaction with acyl-CoA. With 12:0-CoA as donor substrate, the enzyme then had a slight preference for 12:0-LPA over 18:1-LPA. No activity was obtained when the substrates were 12:0-CoA and 12:0-L-α-lysophosphatidyl choline (LPC), but activity with the combination of 18:1-CoA and 18:1-LPC was comparable to that with 18:1-CoA and 18:1-LPA. The medium chain activity, therefore, had the substrate specificity expected of LPAAT, but we could not be certain whether the activity with 18:1 substrates derived from LPAAT, lysophosphatidylcholine acyltransferase (EC 2.3.1.23) or both.

The majority of membrane enzyme solubilizations have used either a bile-salt analogue (e.g. 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonic acid; CHAPS) or polyoxyethylene (e.g. Triton) detergents. We focused on the conditions necessary to achieve efficient solubilization with CHAPS. It was possible to include CHAPS in the assay without loss of activity, provided its concentration did not exceed 0.1%.

Any solubilization procedure, therefore, had to be followed by dilution to restore 0.1% CHAPS and an ionic strength close to that of the standard assay before activity could be measured. With these assay limits defined, we examined pre-assay exposure of the P2 preparation to higher detergent and NaCl concentrations, in order to find the highest concentrations which could be tolerated without irreversible activity loss. The variously treated P2 preparations were centrifuged at 300 000 *g* (max) and the supernatant fractions were diluted and assayed. Thus, the experiment also afforded a preliminary assessment of solubilization. Figure 1 shows the effect of varying the P2 concentration (measured as protein concentration) at fixed CHAPS concentration (expressed as detergent:protein (D : P) ratio), and the consequence of changing the fixed CHAPS level. The NaCl concentration was 1 M throughout. The activity recovered in the supernatant fraction showed little sensitivity to the D : P ratio at low CHAPS concentrations, such as 0.5% (example shown in Fig. 1). However, at high CHAPS concentrations there was a marked dependence on the D : P ratio, suggesting the potential for effective disruption of the membranes. At the higher D : P ratios, 2.25% and 3% CHAPS produced equivalent results, suggesting there was no advantage to be gained from CHAPS concentrations higher than 2.25%. Lowering the NaCl concentration below 1 M reduced the formation of supernatant LPAAT activity at all D : P values tested (data not shown). It is advantageous to keep the detergent and NaCl concentrations as low as possible to minimize the dilution factor required prior to assay. Similarly, very high D : P ratios are to be avoided as they result in excessive dilution of activity. Accordingly, the following NaCl and detergent conditions were adopted for routine production of high-speed supernatant (S3) activity: 1 M NaCl, 2.25% CHAPS and 0.47 mg ml^{-1} P2 membrane protein for a D : P ratio of 48. Typically 50–80% of the original P2 medium-chain LPAAT activity was recovered in the S3 fraction.

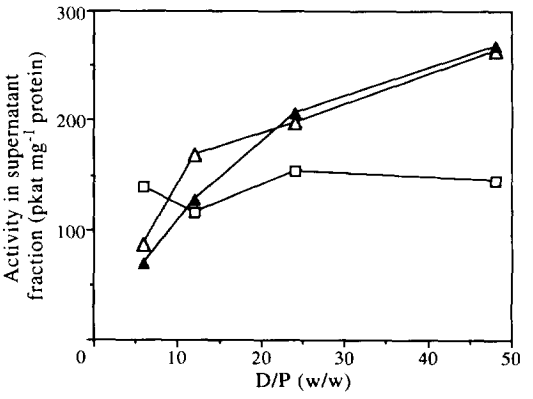

Fig. 1. Effect of altered P2 concentration (expressed as detergent:protein; w : w) and CHAPS concentration in LPAAT solubilization experiments. CHAPS concentrations were 0.5 (□), 2.25 (△) and 3 (▲) %. All activity data normalized to 1 mg original P2 protein. The LPAAT activity of the starting P2 preparation was 615 pkat mg^{-1} protein and the highest supernatant activity obtained represented an apparent solubilization of 58%.

In shifting the LPAAT activity from high-speed pellet to high-speed supernatant fraction, we met the frequently-cited criterion for solubilization of a plant membrane enzyme. However, the buffer now contained considerable NaCl and CHAPS in addition to glycerol and its density might have increased sufficiently to slow the sedimentation of membrane vesicles and give a false-positive indication of solubilization. Our experience in testing a published solubilization method for LPAAT [7] illustrated this possibility, the resulting activity no longer sedimenting at high speeds but still voiding a size-exclusion column. Therefore, two additional criteria were defined in order to test rigorously for solubilization, i.e. native M_r estimation by size-exclusion chromatography and protein fractionation. When the P2 preparation was chromatographed on a Sepharose CL-4B size exclusion column, all the recovered LPAAT activity emerged in the void volume as defined by chromatography of blue dextran (Fig. 2a). In contrast, preliminary size-exclusion chromatography of the S3 preparation showed that the activity behaved as a soluble protein of modest M_r. This was repeated with a column of Superose 12 in order to obtain an M_r estimate (Fig. 2b). (To obtain significant LPAAT activity these fractions had to be assayed with the phospholipid re-association method discussed below.) This change in behavior on size-exclusion columns and the estimated M_r of 44 000 for LPAAT in the S3 preparation, provide strong evidence for solubilization.

If the coconut membranes were solubilized, one would expect the membrane proteins to be at least partially resolvable on chromatography columns. A demonstration of this resolution would eliminate the possibility that the solubilization conditions were merely driving an interaction between membrane vesicles and the matrix material of the size-exclusion column, resulting in a spuriously low estimation of M_r. Sufficient protein resolution occurred on the Superose 12 column to meet this third criterion. As shown in Fig. 2(b), LPAAT activity was resolved from a significant peak of 280 nm-absorbing material. Furthermore, examination of the protein compositions of eluted fractions by SDS-PAGE showed that the elution behaviour of individual proteins differed (Fig. 3). This is consistent with complete dissociation of these proteins from the membrane.

Although pre-mixing of the S3 preparation with phospholipids was not essential for detection of activity, it did result in considerable stimulation (Fig. 4). After chromatography on size-exclusion columns, the activity was almost undetectable unless the fraction aliquots were first mixed with phospholipids. It appears, therefore, that coconut medium chain LPAAT requires the presence of phospholipids for activity, and that native phospholipids present in the S3 preparation were removed on the size-exclusion column. Neither synthetic phospholipids (L-α-phosphatidylcholine, L-α-phosphatidylinositol or L-α-phosphatidylethanolamine, alone or in combination) nor turkey egg yolk phospholipid preparation, provided any significant improvement over the crude soybean L-α-phosphatidylcholine. The concentration dependence of phospholipid stimulation was unaffected by the concen-

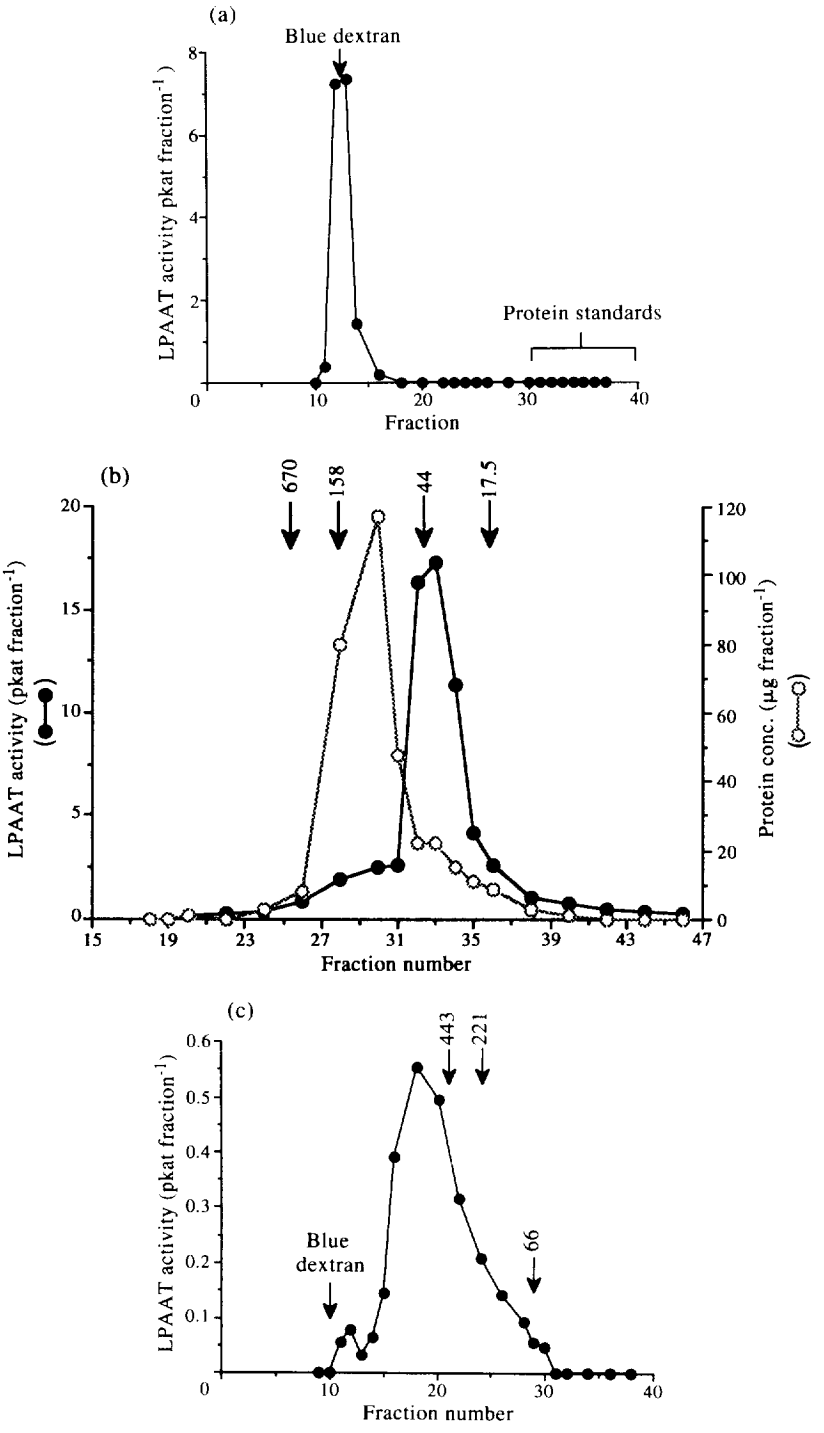

Fig. 2. Chromatography of LPAAT preparations on size-exclusion columns. (a) P2 membrane preparation chromatographed on Sepharose CL-4B. The applied sample contained 21 pkat LPAAT in 200 μl. Fractions of 1 ml were collected with a total activity recovery of 80%. A peak of 280 nm-absorbing material (not shown) co-eluted with the enzyme activity and a larger peak with the unresolved protein standards (M_r 1750–670 000) in fractions 30–40. (b) S3 preparation chromatographed on Superose 12. An S3 preparation was concentrated ten-fold by ultrafiltration, and 200 μl containing 119 pkat LPAAT were applied. The running buffer contained 1% (w/v) CHAPS and 1 M NaCl, these concentrations being essential for good recovery of enzyme activity. Fractions of 0.4 ml volume were collected for an overall activity recovery of 54% (an estimate, as the specific activity may have changed with the switch from native coconut to added soybean phospholipids). The elution of protein standards is indicated by the arrows, along with their M_rs ($\times 10^{-3}$). (c) Phospholipid-activated S3 preparation chromatographed on Sepharose CL-4B. The phospholipid-reactivated S3 preparation contained 4.5 pkat LPAAT in 200 μl. Fractions of 1 ml volume were collected and to assay fraction aliquots it was necessary only to add the substrates. The overall recovery of LPAAT activity was 82%. The elution of protein standards is indicated by the arrows, along with their M_rs ($\times 10^{-3}$).

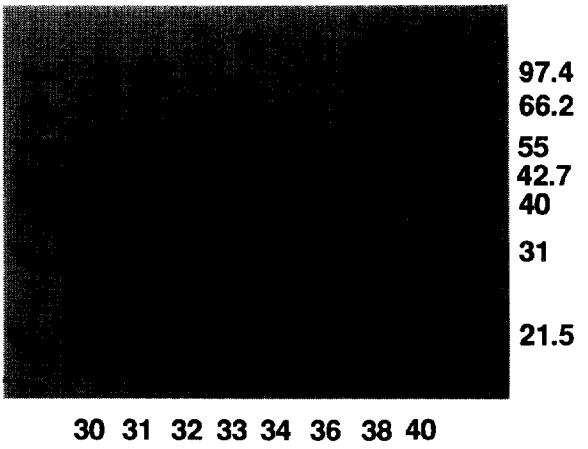

Fig. 3. Protein compositions of fractions from Superose 12 chromatography of solubilized LPAAT resolved by SDS-PAGE. The samples were eluted fractions from the experiment of Fig. 2(b); the protein band detection was by silver-staining. The extreme left and right lanes show standard proteins ($M_r \times 10^{-3}$), the other lanes corresponding to the fraction numbers shown at the bottom of the gel.

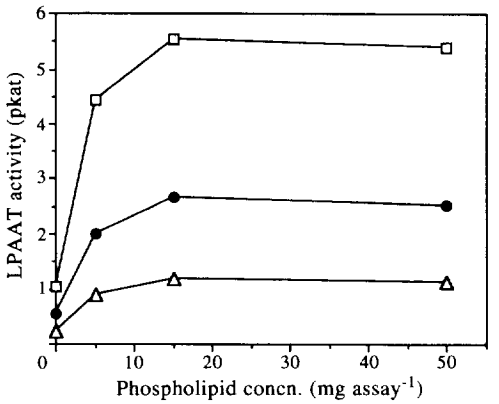

Fig. 4. Effect of soybean phospholipid concentration on reactivation of solubilized LPAAT. The amounts of phospholipids shown, in 1 μl of 0.5% CHAPS, were mixed with 25 μl of an S3 preparation which had first been diluted 5- (□), 10- (●) or 20- (△)fold while maintaining 1% CHAPS and 1 M NaCl. The remainder of the assay followed the standard procedure.

tration of S3 preparation (Fig. 4), 15 μg per assay being optimal. This suggests that the LPAAT concentration was not a limiting factor in the stimulation phenomenon. It was important to provide at least 1 M NaCl in the solubilized LPAAT preparation being mixed with phospholipids. Presumably, the phospholipids were dispersed upon mixing with the high CHAPS and NaCl concentrations of the S3 buffer and then formed vesicles or phospholipid micelles upon dilution with the assay ingredients. Stimulation of LPAAT activity might be explained by incorporation of the enzyme into the artificial membrane vesicles or by stripping of CHAPS from the enzyme into mixed detergent–phospholipid micelles. Size-exclusion chromatography of the phospholipid-activated S3 preparation, under conditions similar to those of the assay, showed a considerable increase in the apparent M_r of LPAAT; a broad peak of LPAAT activity emerged later than the void volume but earlier than that expected for solubilized LPAAT (Fig. 2(c)). This is consistent with the formation of artificial membrane vesicles containing LPAAT, but it is also possible that protein aggregates were formed.

The need to form phospholipid structures is also evidenced by the critical order in which assay components had to be combined to obtain maximum stimulation. Addition of phospholipids just prior to initiating the reaction with 12:0-CoA resulted in only 30% of maximum activity; addition after stopping the reaction was without effect. Therefore, the phospholipids were not acting as simple enzyme or substrate activators, or as enhancers of the post-incubation assay events. However, it was not essential to pre-mix the S3 preparation with the concentrated phospholipids. Pre-mixing of S3 buffer with phospholipids, followed by addition of assay ingredients and substrates to dilute that mixture, and finally addition of S3 preparation, gave 70–100% of the activity obtained with the standard order of additions. Collectively, these observations on the order of assay additions indicate that it was the treatment of the phospholipids rather than the treatment of S3 preparation that was critical for maximum stimulation and activity of LPAAT.

Assay conditions were characterized in most detail for the solubilized preparation after phospholipid activation and using 12:0-containing substrates. Increasing the EDTA concentration to 10 mM, or adding 1 mM Mg^{2+}, Mn^{2+}, or Ca^{2+}, did not affect LPAAT activity. However, activity was reduced by 50% or more if any of these ions was added to 10 mM. Omitting 2-mercaptoethanol (ME) from the assay system resulted in 50% less LPAAT activity and concentrations above 5 mM were inhibitory. Lowering the assay pH from 7.5 to 6.5 resulted in a loss of *ca* 20% of the LPAAT activity. From pH 7.5 to 8.5 the activity was almost unchanged; pH 7.5 was used routinely to minimize non-enzymatic hydrolysis of acyl-CoAs. There was little change in activity with NaCl concentration up to 200 mM, but activity declined at higher concentrations. The activity was insensitive to changes in glycerol concentration in the assay between 5% and 15% (w/v). There was no inhibition or new enzyme activity in the presence of unesterified CoA (40 μM), 12:0-LPC (50 μM), 18:1-LPC (50 μM), or di-12:0-L-α-phosphatidate (PA; 20 μM). Storage of the S3 preparation at 4° overnight was accompanied by up to 50% loss of activity; the loss was much greater at lower glycerol concentrations. There was no loss of activity at 4° if the enzyme was reactivated with phospholipids beforehand. It was possible to store the S3 preparation at − 70° after freezing in liquid nitrogen, with negligible loss of activity upon thawing.

After solubilization from the coconut membranes and reassociation with soybean phospholipids the LPAAT activity retained its specificity for medium chain acyl-CoAs. Exposure of the preparation to 18:1-LPA prior to

addition of phospholipids and assay ingredients no longer caused inhibition but, unlike the situation with 12:0-LPA, the progress curve with 18:1-LPA was non-linear. We, therefore, attempted to compare activities with the two LPAs by measuring initial rates, with the results shown in Table 2. With 12:0-CoA or 18:1-CoA there was a preference for 12:0-LPA over 18:1-LPA. Since the enzyme was reassociated with lipid in order to obtain maximum activity, these preferences may reflect a combination of lipid-solubility of the substrates and enzyme specificity. For this reason, and the complications associated with substrate micellization in the presence of high ionic strength and detergent, we have not attempted to obtain kinetic data. It would also be difficult to compare kinetic parameters for the solubilized and original membrane preparations in any meaningful way. However, the close similarity between the substrate preferences of solubilized, reactivated LPAAT and of the original membrane preparation in our assay system suggests that our solubilization procedure did not compromise enzyme specificity. We therefore believe that we have solubilized LPAAT in a way which will greatly facilitate a more detailed study of its properties, as well as its purification.

EXPERIMENTAL

Tissue extraction and preparation of membrane fraction. Immature coconut seeds (*C. nucifera* L.) were obtained from local retail stores. For max. yield of LPAAT activity it was important to obtain very young specimens referred to as 'green', which have a very pale brown or white endocarp. The endocarp was pierced and the liquid endosperm drained. White endosperm tissue was dissected, the brown testa removed and discarded and the endosperm frozen in liquid N_2 for storage at $-70°$. Prior to extraction, 24 g frozen tissue were powdered by impact crushing in a steel mortar and pestle at liquid N_2 temp. Buffer A (50 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulphonic acid) (HEPES)–NaOH pH 7.5, 3 M NaCl, 10 mM EDTA, 10 mM Na diethyldithiocarbamate (DIECA), 100 μM Pefabloc, 1 μM leupeptin, 0.1 μM pepstatin A, 5 mM ME) at 0–4° was added to the powdered tissue (8 ml g^{-1}) and the mixt. blended with a Polytron homogenizer. The high NaCl concn was critical for effective removal of storage oil. All subsequent steps were performed at 4°.

Table 2. Activities* of phospholipid-reactivated, S3 LPAAT preparations with different substrate combinations

Acyl-CoA substrate†	LPA substrate	% of activity of C_{12} substrates‡ Expt 1	Expt 2
12:0	12:0	(100)	(100)
12:0	18:1	66	54
18:1	18:1	10	7
18:1	12:0	16	9
12:0	none	5	5
18:1	none	0	2

*Initial rates from progress curves.
†Radiolabelled.
‡Experiments 1 and 2 used two different S3 preparations for which the starting activities with 12:0-containing substrates (100%) were 0.2 and 0.8 pkat, respectively.

The resulting crude homogenate was filtered through 4 layers of cheesecloth and centrifuged at 12000 g (max) for 30 min. The floating oil/fat pad and the pellet were discarded, and the supernatant fr. (S1) was filtered through Miracloth. This S1 fr. was then dialyzed overnight against 4 l Buffer B (50 mM HEPES–NaOH pH 7.5, 1 M NaCl, 5 mM ME), with one change of buffer. The dialyzed S1 material was centrifuged at 12000 *g* as described above and the supernatant fr. again filtered through Miracloth. Centrifugation at 100000 *g* (max) for 2 hr generated the membrane pellet, 'P2'. Sufficient Buffer C (50 mM HEPES–NaOH pH 7.5, 200 mM NaCl, 20% (w:v) glycerol, 5 mM ME) was added to the P2 pellet so that, after gentle resuspension in a ground-glass homogenizer, the total vol. was 0.14 ml g^{-1} original tissue powder. This P2 suspension was divided into aliquots, frozen in liquid N_2 and stored at $-70°$.

Solubilization of LPAAT. All steps were carried out at 0–4°. Coconut P2 prepn was dild in Buffer C to a protein concn of 0.94 mg ml^{-1}, determined by the Coomassie dye method [13] with BSA as ref. This suspension was then dild with 1 vol. Buffer D (50 mM HEPES–NaOH pH 7.5, 1.8 M NaCl, 20% (w:v) glycerol, 4.5% CHAPS, 100 μM Pefabloc, 1 μM leupeptin, 1 μM Pepstatin A, 5 mM ME), achieving final concs of NaCl, detergent and protein of 1 M, 2.25% and 0.47 mg ml^{-1}, respectively and a detergent:protein ratio of 48:1 (w/w). The prepn was incubated on ice for 30 min with occasional inversion mixing, then centrifuged at 250000 *g* (max) for 2 hr. The resulting supernatant fr. (S3) was filtered through Miracloth. It could then be stored frozen ($-70°$) with only slight loss of activity.

Substrates. [1-^{14}C]Acyl–CoAs having the acyl groups 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, 18:0 and 18:1 were synthesized enzymatically from free CoA and the corresponding [1-^{14}C]fatty acids (sp. act. typically 50 Ci mol^{-1}) using the method of ref. [14]. Although developed for long-chain acyl-CoAs, this method gives high yields and purity down to the 6:0 thioester. Acyl-CoAs were stored as 0.2–0.5 μM solns in MeOH at $-70°$ and used exclusively for specificity determinations. [1-^{14}C]12:0-CoA and [1-^{14}C]18:1-CoA used for routine assays were prepd in the same manner but the labelled fatty acid was first dild with unlabelled 12:0 or 18:1 to a sp. act. of 20 Ci mol^{-1}, as an economy measure. These acyl-CoAs were dild to 125 μM and stored in 3 mM Na acetate (pH 4.8) at $-70°$. 18:1-LPA was obtained from Sigma. 12:0-LPA was synthesized by the method of ref. [15] using phospholipase D to cleave choline from commercially available (Sigma) 12:0-LPC. The action of phospholipase A2 on 12:0-PA [3] gave much lower yields. [1-^{14}C]12:0–ACP was prepd as described previously [16].

Enzyme assays. Assays of crude homogenate and P2 (membrane) prepns for LPAAT activity were based on ref. [3]. P2 (20 μl) was added to 218 μl assay mixt. in a 4-ml, screw-cap vial. The assay mixt. provided the following conditions in the final 250 μl assay (in addition to contributions from the enzyme sample): 100 mM HEPES–NaOH (pH 7.5), 200 mM NaCl, 5% (w : v) glycerol, 10 mM EDTA and 5 mM ME. 12 : 0- or 18 : 1-LPA substrate was added (2.5 μl) for a final concn of 20 μM. Blanks were included in which LPA was omitted. Incubation was started by adding 10 μl 125 μM radiolabelled 12 : 0-CoA or 18 : 1-CoA for 5 μM final concn and continued for 20 or 30 min at 30°. These acyl-CoA and LPA concns were below satn for LPAAT, to conserve substrates and facilitate the determination of specificity. Termination was by addition of 0.25 ml 1 M KCl in 0.2 M H_3PO_4. BSA (1 mg ml^{-1}, 40 μl) and 0.75 ml of 67 μg ml^{-1} PA in $CHCl_3$–MeOH (2 : 1) were then added (PA acyl groups both 12 : 0 or 18 : 1 as appropriate). Upon thorough mixing, the radiolabelled PA product partitioned into the organic phase. The vial was centrifuged briefly at low speed, the aq. (upper) phase discarded and the total radioactivity extracted into the organic phase determined by liquid scintillation counting; 100 μl organic phase were transferred to a 20 ml-vial and the solvent evapd with a stream of warm air before addition of 3 ml Aquasol scintillation fluid. Not all the organic phase radioactivity was associated with PA product; free fatty acid from limited acyl-CoA hydrolysis and small amounts of unreacted acyl-CoA, were also present. A further 100 μl of organic phase was therefore applied to a silica gel TLC plate (0.25 mm layer). Ascending chromatography was carried out for 50 min with $CHCl_3$–pyridine–88% formic acid (50 : 30 : 7). Radioactive zones were visualized and quantitated using an imaging system (Ambis Systems). R_fs of the di-12 : 0- and di-18 : 1-PA were 0.67 and 0.71, respectively; PAs having mixed acyl groups migrated with intermediate R_fs. Radiochromatogram data were used to calculate a ratio of PA radioactivity to total TLC lane radioactivity for each sample. The quantity of PA produced by LPAAT was then calculated from the product of this ratio and the total radioactivity of the 450 μl organic extract which had been determined by scintillation counting. In the standard assay employing 12 : 0-containing substrates, the progress curve was typically linear for at least 20 min and the rate of PA formation was proportional to the concn of P2 prepn up to 0.5 pkat activity. The assay with 12 : 0-ACP as donor substrate was conducted identically, after checking that there was no significant hydrolysis of the 12 : 0-ACP by the P2 preparation.

When assaying S3 preparation and frs from size-exclusion columns, the first soln placed in the assay vial was 1 μl 50 mg ml^{-1} crude soybean phospholipids (crude L-α-phosphatidylcholine; Sigma 'Type IVs'; PL) previously dispersed in 0.5% CHAPS by sonication. The LPAAT sample was dild with 50 mM HEPES–NaOH pH 7.5, 20% (w : v) glycerol and 5 mM ME, to reset the CHAPS and NaCl concns to 1% and 1 M, respectively; 20 μl were added to the PL in the assay vial with mixing. The assay ingredients and substrates were added next as described above, further diluting the CHAPS, NaCl and PL, and the assay was continued as before. The S3 progress curve was linear for at least 20 min, and LPAAT activity was proportional to S3 concn up to 0.5 pkat activity.

Determinations of acyl-CoA specificity used the chain length series of radiolabelled acyl-CoAs stored in MeOH. The required vol. of each acyl-CoA for 5 μM final concn was first placed in the assay vial and the MeOH evapd under N_2. The next soln added was the 218 μl assay mixt., which also contained 1 μl 125 mg ml^{-1} crude soybean PL in 2-methoxyethanol. The shorter-chain acyl-CoAs could not be redissolved completely from the vial surface with the detergent-containing assay and enzyme buffers alone, but the inclusion of soybean PL ensured quantitative recovery. The reaction was started by the addition of appropriately dild (and PL-treated) P2, S3 or column fr. LPAAT prepns and continued as before.

All assays were conducted in duplicate and the mean activity was corrected for the LPA-independent background value (except for the data shown in Table 2). This is an over-correction as the activity which was independent of added LPA was probably using acceptor substrates in the coconut membranes or the soybean PL prepn. Additional evidence that the radioactive product migrating with authentic PA on the TLC plate resulted from LPAAT action was obtained by treating it with phospholipase A2 [17]; the PA-associated radioactivity was converted quantitatively into a product which co-migrated with free fatty acids.

Column chromatography. Size-exclusion chromatography of the P2 prepn was conducted on a 20 × 1.5 cm column of Pharmacia, Sepharose CL-4B, equilibrated with Buffer C at 0.2 ml min^{-1}. With the S3 prepn, we used a 30 × 1 cm Pharmacia Superose 12 column equilibrated with 50 mM HEPES–NaOH pH 7.5, 1 M NaCl, 20% (w : v) glycerol, 1% CHAPS and 5 mM ME at 0.1 ml min^{-1}. For the PL-activated S3 prepn, the 20 × 1.5 cm column of Sepharose CL-4B was equilibrated with 100 mM HEPES–NaOH pH 7.5, 280 mM NaCl, 7% (w : v) glycerol, 10 mM EDTA, 0.08% CHAPS, 5 mM ME and 0.2 mg ml^{-1} crude soybean PL. This column buffer was prepd by diln of the soybean PLs according to the assay procedure, so that detergent, NaCl and PL concn corresponded to those in the incubation phase of the assay. The flow rate was 0.2 ml min^{-1}. Protein standards were thyroglobulin (M_r 670 000), equine apoferritin (M_r 443 000), bovine γ-globulin (M_r 158 000), BSA (M_r 66 000), chicken ovalbumin (M_r 44 000) and equine myoglobin (M_r 17 500). They were chromatographed under the same conditions as the coconut prepns and gave the expected linear relationship between log M_r and relative migration rate.

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