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Substrate selectivity of plant and microbial lysophosphatidic acid acyltransferases

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Abstract

Linoleic acid (18:2) is found in a large variety of plant oils but to date there is limited knowledge about the substrate selectivity of acyltransferases required for its incorporation into storage triacylglycerols. We have compared the incorporation of oleoyl (18:1) and linoleoyl (18:2) acyl-CoAs onto lysophosphatidic acid acceptors by sub-cellular fractions prepared from a variety of plant and microbial species. Our assays demonstrated: (1) All lysophosphatidic acid acyltransferase (LPA-AT) enzymes tested incorporated 18:2 acyl groups when presented with an equimolar mix of 18:1 and 18:2 acyl-CoA substrates. The ratio of 18:1 to 18:2 incorporation into phosphatidic acid varied between 0.4 and 1.4, indicating low selectivity between these substrates. (2) The presence of either stearoyl (18:0) or oleoyl (18:1) groups at the *sn*-1 position of lysophosphatidic acid did not affect the selectivity of incorporation of 18:1 or 18:2 into the *sn*-2 position of phosphatidic acid. (3) All LPA-AT enzymes tested incorporated the saturated palmitoyl (16:0) acyl group from equimolar mixtures of 16:0- and 18:1-CoA. The ratios of 18:1 to 16:0 incorporation are generally much higher than those of 18:1 to 18:2 incorporation, varying between 2.1 and 8.6. (4) The LPA-AT from oil palm kernel is an exception as 18:1 and 16:0 are utilised at comparable rates. These results show that, in the majority of species examined, there is no correlation between the final *sn*-2 composition of oil or membrane lipids and the ability of an LPA-AT to use 18:2 as a substrate in in vitro assays.

Keywords: Lysophosphatidic acid acyltransferase; Plant; Yeast; Acyl-CoA; Substrate selectivity

1. Introduction

In developing plant seeds, triacylglycerol (TAG) synthesis is classically believed to occur via the acyl-CoA-dependent Kennedy pathway, in which acyl groups are sequentially transferred from acyl-CoA substrates to the *sn*-1, 2 and 3 positions of glycerol-3-phosphate (Ohlrogge and Browse, 1995). These acylation reactions are catalysed by separate acyltransferase enzymes, which are thought to be located in specific micro-domains of the endoplasmic reticulum (Murphy and Vance, 1999) and TAG is finally exported to storage particles called lipid- or oil-bodies (Huang, 1992). The substrate selectivity of acyltransferases, and in particular that of the acyl-CoA:lysophosphatidate acyltransferase (LPA-AT; EC 2.3.1.51) present in seeds, is known to be an important factor in determining the final acyl composition of some plant oils (Bernerth and Frentzen, 1990) and transfer of such enzymes between plant species can result in the production of novel TAG molecules (Brough et al., 1996; Lassner et al., 1995).

The properties and consequent commercial value of an oil are determined by its acyl composition. The amount of linoleate (18:2) in an oil is important because it is prone to oxidative decomposition leading to spoilage and there have been attempts to lower the amount of 18:2 in some oil crops with breeding programs (Purdy, 1986). To date, however, there is little information on the utilisation of linoleoyl-CoA by acyltransferases in developing oil seeds, although it has been used as a substrate in LPA-AT assays with seeds of safflower and turnip rape (Ichihara et al., 1987; Bafor et al., 1990) and pea shoots (Hares and Frentzen, 1991). These studies mostly used single substrate assays, containing one acyl-CoA species in reaction mixtures.

We are interested in the selectivity between and possible discrimination against 18:1- and 18:2-CoAs by LPA-ATs from seeds which accumulate large levels of storage lipids. Knowledge of acyltransferase substrate selectivity obtained from in vitro assays may be beneficial for biotechnological applications concerned with the modification of seed oil composition, and the aim of this work was to identify a source for an enzyme which

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discriminates against 18:2. Here we report the results of selectivity assays using 18:1- and 18:2-CoA and enzyme samples from a number of plant and microbial species. Our initial experiments were restricted to microsomal pellets, which have been the most commonly used cell fraction for the study of TAG biosynthesis in developing seeds e.g. Laurent and Huang, 1992; Oo and Huang, 1989; Cao et al., 1990; Murphy and Mukherjee, 1987. However, since high LPA-AT activity was noted in the lipid body fraction of several species, studies were extended to include these and a comparison of the selectivity in the two fractions carried out.

Selectivity was determined using differentially radiolabelled acyl-CoAs in a competitive assay. In addition to looking at 18:1 versus 18:2 selectivity, the assay was used to assess LPA-AT activity with saturated acyl-CoAs (16:0- and 18:0-CoA). The potential importance of observed selectivity versus the final acyl composition of an oil is discussed.

2. Results

2.1. Assay development and precipitation of lysophosphatidic acid substrates

Initial testing of the assay system was carried out under non-competitive conditions using ^{32}P-labelled 18:0- or 18:1-lysophosphatidic acid (LPA) as an acceptor. This method has the advantage that the product can be readily detected and quantified by phosphor-imager analysis after separation on TLC plates, significantly speeding up data acquisition. Experiments to investigate both the extent and linearity of the LPA-AT reaction were carried out to optimize acyl-CoA incorporation onto both LPA substrates. These assays were performed with *E. coli* microsomes and reaction mixtures were based on those used previously in the laboratory (Brown et al., 1995). They included 0.5 mM $MgCl_2$ in the assay mix together with other constituents as in Experimental.

Using oleoyl-LPA as the acyl-acceptor under these conditions, more than 90% of the LPA was converted to phosphatidic acid (PA) in extended incubations. In assays using either 16:0- or 18:0-LPA as acyl acceptors however, there was a marked deviation from linearity with respect to amount of protein added and a decrease in the extent of the reaction. In an assay containing 18:0-LPA and 18:1-CoA as substrates, the reaction essentially came to a halt after only 10–15% of the substrate was consumed (Fig. 1A). Subsequent experiments to try and optimise incorporation with saturated LPA showed that this effect was due to precipitation of the LPA by Mg^{2+} ions, effectively reducing the substrate concentration. This is shown by the LPA becoming readily sedimented by centrifugation at 13,000 *g* in the presence of magnesium and the reversal of this upon addition of EDTA (Fig. 1B). Similar effects have been reported for long chain saturated acyl-CoA (Constantinides and Steim, 1986) but the observation has not previously been made with LPA. Omission of $MgCl_2$ from the reaction mix considerably reduced the precipitation, while addition of 2 mM EDTA completely eliminated the problem and restored linear reaction rates. EDTA was therefore included as a standard component in all subsequent assays. All acyl-CoAs used seemed fully soluble in the assay mix used as judged by non-sedimentation during centrifugation (5 min at

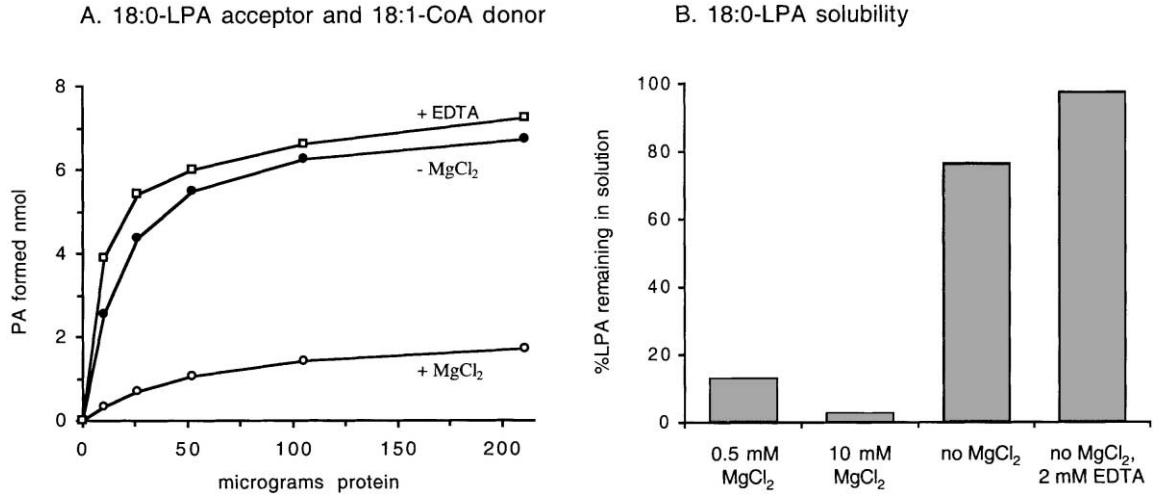

Fig. 1. (A) Effect of $MgCl_2$ on the LPA-AT reaction with 18:0-LPA. The amount of PA formed by increasing quantities of *E. coli* microsomal protein was determined in the presence (open circles), and absence (closed circles) of 0.5 mM $MgCl_2$, and in an assay containing no $MgCl_2$ and supplemented with 2 mM EDTA (open squares). Donor (18:1-CoA) and acceptor substrates were present at 100 μM and the reaction was allowed to proceed for 3.5 min at 30 °C. Other conditions were as in Experimental. (B) Effect of $MgCl_2$ on the solubility of 18:0-LPA. ^{14}C-labelled 18:0-LPA (100 μM initial concentration) was incubated for 30 min (room temperature) in assay mixture containing indicated amounts of $MgCl_2$ and EDTA. Results are expressed as the percentage of the original counts remaining in solution following centrifugation for 15 min at 13,000 *g*.

13,000 *g*), and also by the fact that the substrate could be almost completely consumed during extended incubations. Experiments investigating the effect of Mg^{2+} on reactions containing 18:1-LPA showed that this acceptor was not prone to precipitation.

The addition of proteins that can bind acyl-CoAs, such as BSA or acyl-CoA binding protein (ACBP), can stimulate acyltransferase activity (Bertrams and Heinz, 1976; Brown et al., 1998). The effect of adding either BSA or ACBP to the LPA-AT assay was studied using non-competitive assays with 18:1-LPA and 18:1 acyl-CoA. In assays with sunflower microsomes, the addition of either protein stimulated phosphatidic acid formation (Fig. 2). At higher concentrations both proteins caused increasing inhibition of enzyme activity, probably by competing for substrate. The same effect has been reported before with ACBP additions to glycerol-3-phosphate acyltransferase assays (Brown et al., 1998). Because BSA was commercially available we decided to use this protein in our assays. BSA was included in all reaction mixes at a concentration of 1 mg/ml (15 μM)

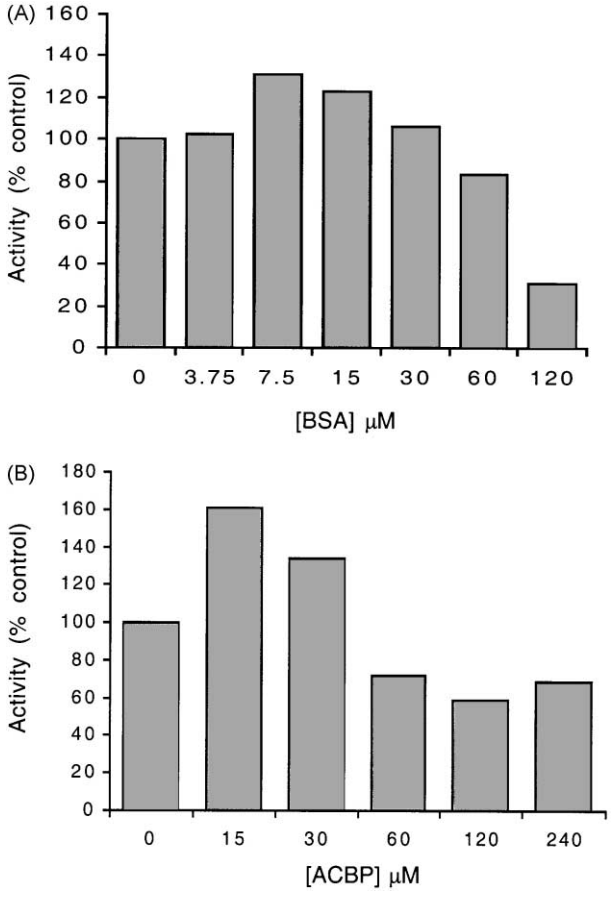

Fig. 2. Effect of (A) BSA and (B) ACBP on the activity of sunflower LPA-AT. Activities were measured in the presence of 100 μM 18:1-LPA, 100 μM 18:1-CoA and the indicated concentrations of BSA and ACBP. Activities are expressed as a percentage of the activity measured in the absence of BSA or ACBP. Other assay conditions were as in Experimental.

and significantly increased measurable acyltransferase activity (up to 2-fold depending on enzyme source). The stimulatory effect of BSA in the LPA-AT assay is probably bi-factorial. Firstly, by binding acyl-CoAs it prevents micelle formation and keeps these substrates available to the enzyme and in doing this also guards the microsomes against the detergent effect of these substrates, which would otherwise cause lysis and enzyme inactivation (Lichtenstein et al., 1982; Lichtenberg et al., 1983). In tests with rapeseed microsomes, no difference was observed in selectivity with 18:1/18:2 and 18:1/16:0 equimolar acyl-CoA mixtures whether or not BSA was present. It was therefore presumed not to selectively bind any particular acyl-CoA and hence alter the apparent ratio of acyl-CoAs available for phosphatidic acid formation.

2.2. LPA-AT activity in different species and its distribution between microsomal and fat pad fractions

The specific activity of LPA-AT in different species was determined using the [^{32}P]-LPA assay system with 18:1-CoA as an acyl donor. 18:1-, 18:0- and 16:0-LPAs were each tested as acceptor species. All substrates were present at 100 μM, a concentration which was found to give maximal activities in test assays. Initial studies concentrated on microsomal fractions, since the endoplasmic reticulum is presumed to be the major site of TAG biosynthesis. However, as significant activities were also found to be associated with the fat pad of most species investigated, the study was later extended to include this fraction (Table 1). Data for the microsomal fractions only is shown if homogenisation and centrifugation produced no, or only a very small, floating fat pad. Although other researchers have also noted the presence of significant acyltransferase activity in lipid body fractions from various sources (e.g. Kamisaka et al., 1997; Martin and Wilson, 1984; Oo and Chew, 1992), the relationship between this and the microsomal activity has not been thoroughly investigated and the former is often ascribed to 'contamination'.

To try and minimise the possibility of contamination with other cellular membranes, more highly purified fat pad fractions were prepared from rape, sunflower and *Limnanthes*. Purification was effected by repeated washing with homogenisation buffer as described in Experimental. The total and specific activities associated with the fat pad fractions following three washing cycles are shown in Table 1. The rapeseed fat pad was also washed with 0.01% Triton X-100 or KCl up to 1 M before recentrifugation without removal of LPA-AT activity, implying that under this set of conditions the activity associated with the fat pads cannot be removed by mild solubilisation and centrifugation.

In rape and *Limnanthes*, the total amount of LPA-AT activity in washed fat pads was some 200× greater

Table 1
LPA-AT activity in fat pad and microsome fractions of different species

Material	Fraction	Specific activity (nkat/g protein)			Total activity (pkat/g fresh material)
		18:1-LPA[a]	18:0-LPA[a]	16:0-LPA[a]	18:1-LPA[a]
Avocado	Microsomes	200	208.3	171.6	48.7
Avocado	Fat pad	300	–	–	270
*Candida*107	Microsomes	126.6	63.3	60	–
Cocoa	Microsomes	290	180	138.3	141
E. coli	Microsomes	746.7	678.3	901.6	–
Limnanthes	Microsomes	9.5	–	–	3.7
Limnanthes	Fat pad[b]	305	–	–	761.3
Oil palm kernel	Microsomes	37.5	44.5	–	11.2
Oil palm kernel	Fat pad	23.3	–	–	21.5
Rapeseed	Microsomes	15.5	12.8	13.8	1.1
Rapeseed	Fat pad[b]	587.2	–	–	252.5
S. cerevisiae	Microsomes	35	11.5	14.8	–
Shea	Microsomes	0.5	–	–	0.2
Shea	Fat pad	55.3	38.3	39.5	93
Simarouba	Microsomes	706.6	200	–	183.3
Simarouba	Fat pad	471.6	260	–	185
Sunflower 24 DAF	Microsomes	696.6	415	428.3	337.7
Sunflower 27/28 DAF	Microsomes	216.2	–	–	54.7
Sunflower 27/28 DAF	Fat pad[b]	14.8	–	–	58

[a] Acyl acceptor. All rates were determined with 18:1-CoA as donor, with both substrates present at 100 μM.
[b] Washed fractions. Dashes indicate values not determined.

than in microsomes, despite the fact that the fat pad figure is likely to be an underestimate due to the difficulty in recovering this entire fraction during preparation. The specific activity was also some 30× greater in the lipid fraction. In contrast sunflower microsomes and fat pads contained comparable amounts of LPA-AT activity, with the specific activity being higher in the former.

All enzyme preparations tested were able to use saturated and unsaturated LPA substrates at similar rates (in the absence of magnesium) showing there is no absolute specificity for certain acyl acceptors.

The single substrate assays were important to identify fractions which were biologically active and determine the quantity of each fraction to add to subsequent assays. We were next interested in using dual substrate competitive assays, where both substrates were present in the reaction mixture, to investigate the possible substrate selectivity of the enzymes.

2.3. Acyl-CoA selectivity of lysophosphatidic acid acyltransferases

LPA-AT selectivity was assayed for all enzyme sources with both 18:1/18:2- and 18:1/16:0-CoA acyl-donor mixtures, using both 18:0- and 18:1-LPA as the acyl-acceptor. There was some difficulty measuring initial reaction velocities because of deviation from standard linear kinetics. For each time point of an assay, the amount of 18:1-CoA incorporated into PA is divided by the amount of the other substrate incorporated. An example data set, using *Limnanthes* microsomes and a labelled 18:1/18:2-CoA mixture is shown in Fig. 3. The ratios from this graph are 0.65, 0.64 and 0.65 at 2.5, 5 and 10 min respectively. For each assay, the average of the three ratios obtained at different time points was calculated and the results are shown in Table 2, together with the number of replicate assays. Selectivity ratios were extremely reproducible between experiments. We discuss salient features of this table below.

2.3.1. Selectivity with 18:1 LPA as an acyl acceptor

None of the species investigated showed a high degree of discrimination between 18:1- and 18:2-CoA when presented as an equimolar mix: i.e. none of the enzymes tested showed an incorporation ratio of greater than 2 (oleate-preferring), or significantly less than 0.5 (linoleate-preferring). Differences in the ratio of oleate and linoleate incorporated from an equimolar mixture were evident between species (Table 2, column 3). Avocado, *Limnanthes* and sunflower showed a consistent preference for 18:2-CoA (ratio <1) whereas slight preferences for 18:1-CoA were exhibited by others. The majority of enzyme preparations tested here displayed ratios close to 1. In comparison, most of the species studied showed significant discrimination against 16:0-CoA when presented in an equimolar mix with 18:1-CoA, with specificity ratios generally exceeding 2 (Table 2, column 4). The one exception to this was the enzyme from oil palm kernel, which accepted 16:0-CoA almost as readily as 18:1-CoA.

Table 2
Selectivity of fat pad and microsomal LPATs. The ratios[a] of acyl-CoA incorporation from equimolar (10 μM each) mixes of the indicated acyl-CoAs are shown

Material	Fraction	18:1-LPA[b]			18:0-LPA[b]	
		18:1/18:2[c]	18:1/16:0[c]	18:1/18:0[c]	18:1/18:2[c]	18:1/16:0[c]
Avocado	Microsomes	0.70±0.12 (5)	2.21±0.39 (3)	3.2 (1)	0.99 (1)	1.98 (1)
Avocado	Fat pad	0.47±0.07 (5)	–	–	–	–
Candida	Microsomes	1.24±0.13 (3)	2.84±0.58 (3)	–	1.11 (1)	2.33 (1)
Cocoa	Microsomes	1.13±0.29 (3)	4.69±1.05 (3)	–	0.98 (1)	5.42 (1)
E. coli	Microsomes	1.38±0.29 (3)	5.78±0.50 (3)	–	1.17 (1)	5.53 (1)
Limnanthes	Microsomes	0.65±0.05 (4)	8.34±0.22 (3)	–	–	–
Limnanthes	Fat pad	0.62±0.04 (4)	8.61±0.93 (3)	–	–	–
Oil palm kernel	Microsomes	1.32±0.15 (3)	1.06±0.02 (3)	1.01 (2)	1.13 (1)	1.22 (1)
Rapeseed	Microsomes	0.92±0.11 (3)	5.10±1.18 (3)	4.88(2)	1.14 (1)	6.42 (1)
Rapeseed	Fat pad	0.93±0.17 (3)	–	–	–	–
S. cerevisiae	Microsomes	1.06±0.14 (3)	2.09±0.50 (3)	–	0.94 (1)	2.35 (1)
Shea	Fatpad	1.06±0.13 (3)	2.78±0.61 (3)	–	1.06 (1)	2.46 (1)
Simarouba	Microsomes	1.17±0.21 (3)	4.77±0.93 (3)	–	1.57 (1)	5.72 (1)
Sunflower (27/28 DAF)	Microsomes	0.44±0.09 (3)	4.65±1.44 (3)	–	0.74±0.04 (3)	4.54 (1)
Sunflower (27/28 DAF)	Fat pad	0.48±0.01 (3)	–	–	–	–

[a] The data is presented as mean values±S.D., *n* in parentheses. Dashes indicate values not determined.
[b] Acyl acceptor.
[c] Acyl-CoAs.

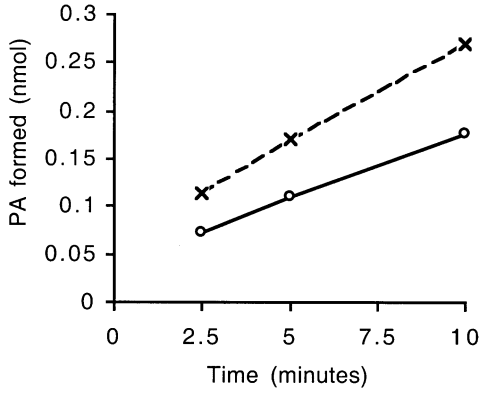

Fig. 3. Selectivity assay using *Limnanthes* microsomes and a mixture of 18:1 and 18:2 acyl-CoAs. The graph shows the incorporation into phosphatidic acid of 18:2 (dashed line) and 18:1 (solid line) from an equimolar mixture of 18:1- and 18:2-CoA, using 18:1-LPA as the acyl acceptor.

To investigate whether the basis for discrimination against 16:0 incorporation was the chain length, or the degree of saturation of the substrate, selectivity assays were also carried out for some species using 18:0-CoA (Table 2, column 5). In all cases the behavior with 18:0-CoA closely resembled that with 16:0-CoA, with avocado and rapeseed discriminating strongly against the saturated compound as a substrate (ratios >3) but oil palm accepting it almost as readily as 18:1-CoA. This result indicates that it is the presence of an unsaturated bond in the acyl chain, rather than the chain length of the acyl group which affects catalytic activity.

2.3.2. Selectivity with different acyl-acceptors

We investigated the effect of a different acyl acceptor in case this altered the observed acyl-CoA selectivity. Saturated 18:0-LPA was used in competitive assays, with mixtures of 18:1/18:2 or 18:1/16:0 acyl-CoAs. The selectivity ratios obtained with 18:0-LPA as an acceptor were essentially identical to those obtained with 18:1-LPA (Table 2, columns 6 and 7). This suggests that, at least for these substrates and the enzyme sources tested, the acyl group at the *sn*-1 position of the glycerol backbone does not have a significant influence on what is incorporated at the *sn*-2 position. Clearly, further experiments would be required to confirm that this is true of all acyl acceptors.

2.4. Selectivity of fat pad and microsomal lysophosphatidic acid acyltransferases

In an attempt to try and establish whether acyltransferase activities in the fat pad and microsomes represent the same or different enzymes, selectivity assays were carried out using 18:1/18:2-CoA. For sunflower, rape and *Limnanthes* there was essentially no difference between the selectivity ratios obtained for fat pads and the microsomes, suggesting it is the same LPA-AT(s) being assayed in each fraction. In avocado however, the fat pad showed a slight but consistently greater preference for 18:2- over 18:1-CoA than did the microsome fraction. The small observed differences could indicate there are different enzymes in these two fractions, although they could also be brought about by differences in the micro-environments of the same enzyme.

3. Discussion

A number of factors contribute to the final stereospecific composition of storage triacylglycerols. Major, easily identifiable parameters are: the composition of the acyl-CoA pool available for TAG biosynthesis, post-synthetic re-modelling of synthesized TAG molecules, and the specificity of acyltransferases for particular acyl donors and acceptors. The relative importance of each of these will vary according to the plant species. A major aim of this research was to assess the importance of LPA-AT substrate selectivity in determining the final ratio of 18:1 to 18:2 at the *sn*-2 position of storage triacylglycerols. This is of particular interest to the food industry, as 18:2 is much more readily oxidised to unwanted side products than 18:1. As we have screened a wide variety of plants it has only been possible to look at the major activity in relevant tissues. Few LPA-ATs have been cloned and overexpressed from plants, allowing selectivity assays to be done with individual enzymes, and this study was concerned with identifying a source of enzymes with low 18:2 activity.

To study the influence of LPA-AT selectivity on *sn*-2 18:2 levels, selectivity assays with mixed 18:1- and 18:2-CoAs were carried out on enzyme preparations from a number of plant and microbial sources. These included plants which may contain an LPA-AT that discriminates against 18:2, as the proportion of this fatty acid at the *sn*-2 position is less than that found in the total TAG. Some of the microbial enzyme sources, such as *E. coli* and *S. cerevisiae* do not normally synthesize 18:2 and were thought good candidates to contain an LPA-AT that would discriminate against this fatty acid.

Crude microsomal or fat pad fractions were used in LPA-AT assays. No attempt was made in this study to separate material into strictly defined membrane fractions because plant tissue was stored at −80 °C on arrival, making attempts at sub-cellular fractionation problematic due to freezing damage. These experiments were carried out to assess the potential importance of LPA-AT selectivity in determining the final composition of triacylglycerols and all plant material used was of the correct age to be actively synthesizing oil. The major LPA-AT activity detected therefore will be that associated with TAG rather than membrane biosynthesis. A correlation between the fatty acid composition of an oil and the results from LPA-AT assays with crude microsomal fractions has been shown in developing rape-seed, where it was demonstrated erucic acid (22:1) could not be incorporated into PA and is not present at the *sn*-2 position of the oil (Oo and Huang, 1989).

To date, most studies of acyltransferase specificity and selectivity have concentrated on a limited range of acyl-donors, commonly comparing those of different chain length (especially 22:1-, 18:1-, 16:0- and 12:0-CoA), while groups of the same chain length but different degrees of saturation have been little compared. Studies which have been performed using 18:2 substrates have also used different experimental conditions, making comparison of results difficult. We therefore wanted to set up a standard competitive assay for analysis of all enzymes preparations. It was important to check all substrates were freely available in assay mixtures, and initially we studied the effect of substrate concentration and linearity with respect to added protein using [^{32}P]-labelled LPAs in single substrate assays. This demonstrated a problem of precipitation of certain LPAs by magnesium, which had not been reported before. Our results suggest that magnesium should not be added to acyltransferase assays, particularly if saturated LPAs are to be used, and the reported acyltransferase activity of oil palm kernel towards 12:0-LPA with 1 mM Mg present (Oo and Huang, 1989) may have been affected by this.

Using differentially labelled acyl-CoAs in competitive assays, it was relatively straightforward to simultaneously follow the rate of incorporation of two acyl groups onto a defined LPA acceptor. Experiments were done with both 18:1- and 18:0-LPA to determine if the nature of the acyl acceptor influenced LPA-AT selectivity, but no differences were observed using 18:1/18:2, or 18:1/16:0 acyl-CoA mixtures for any enzyme.

All the enzymes tested were able to incorporate both 18:1 and 18:2 acyl groups at significant rates. This is despite the fact that several of the plant species do not accumulate significant quantities of 18:2 at the *sn*-2 position of their storage TAG (Table 3) and *S. cerevisiae* or *E. coli* do not usually contain 18:2 fatty acid. The species chosen can be split into three different classes: (1) those with the majority of fatty acids at the *sn*-2

Table 3
Occurrence (mol%) of palmitate, stearate, oleate and linoleate at the *sn*-2 position of plant triacylglycerols

	Fatty acid			
	16:0	18:0	18:1	18:2
Avocado mesocarp[a]	5.0	–	70.0	21.0
Cocoa[ab]	1.7	2.1	87.4	8.6
Limnanthes douglasii[c]	1.3	0.7	2.8	1.4
Oil palm kernel[b]	2.9	0.3	11.8	1.3
Rapeseed[b]	0.6	–	37.3	36.1
Shea[d]	0.6	4.9	82.0	12.0
Simarouba[e]	0.3	0.8	94.9	2.8
Sunflower[b]	1.0	–	23	7.6

[a] J. Am. Oil Chem. Soc. 49 (1972) 229–232.
[b] The Lipid Handbook. Chapman and Hall, London (1994).
[c] Christie, W.W. personal communication.
[d] Oleagineaux 34 (1979) 405–409.
[e] Unilever analysis.

position being 18:1 and 18:2 (with the ratio between the two varying greatly), (2) those in which the minority are these two groups, such as *Limnanthes* and oil palm kernel and (3) those which do not contain any 18:2. Our results show no clear correlation between the amount of 18:2 or 18:1 at the *sn*-2 position of major TAGs or membrane lipids and the major LPA-AT activity towards these substrates, shown by the ratio of their incorporation into PA (Table 2).

The amount of 18:2 in a storage oil therefore appears to be more influenced by the activity of oleate desaturase, factors affecting the availability of acyl-CoAs and the extent to which TAGs are modified after synthesis. Such TAG modification after synthesis has been reported in safflower and castor seeds (Stobart et al., 1997; Mancha and Stymne, 1997). In safflower, transacylation reactions between TAG and monoacylglycerol form diacylglycerol molecules, and these can be converted to phosphatidylcholine by cholinephosphotransferase. These reactions therefore provide a way of making acyl-groups in TAG available for further desaturation (which occurs on phosphatidylcholine) leading to enrichment of the oil with linoleate. The relative importance of acyl-CoA pool composition and remodelling of TAGs is likely to vary between species.

Our results demonstrate the ability of LPA-ATs to incorporate certain acyl groups, for example 18:2, which are not abundant in the membrane lipid or TAG normally present in a given organism. It can be anticipated from the result of these studies that in species such as oil palm and cocoa there is no restriction caused by the endogenous LPA-AT on the incorporation of 18:2 or 18:1. Hence the introduction of appropriate desaturases into these plants would be expected to result in TAGs containing high levels of linoleate at the *sn*-2 position.

These assays hoped to identify an average or dominant LPA-AT in plant seeds or microbial tissues that discriminated against 18:2-CoA, with a view to engineering acyltransferases such that the amount of 18:2 at the *sn*-2 position in oils was lowered. Our results show that in the tissues examined such an enzyme does not exist, and the exploitation of such technology to change the amounts of 18:2 seems unlikely. There may be sound physiological reasons for this as C18 fatty acids are important membrane constituents and LPA-AT enzymes are required for their incorporation.

From this study it can be concluded that in a wide variety of species there is little selectivity between 18:1- and 18:2-CoA donor substrates, but 16:0 and 18:0 are selected against. One notable exception is oil palm kernel where 18:1- and 16:0-CoA are utilised at the same rate under competitive conditions. The evaluation of the substrate specificity of acyltransferases towards other substrates will have to be performed on a case-by-case study to come to conclusions of their preferred selectivity, and this is probably best achieved using competitive assays. In certain species the final composition of storage triacylglycerols will be determined by substrate selectivity and in others by availability of substrate and remodelling events.

4. Experimental

4.1. General

[γ-^{32}P]-ATP (10 Ci/mmol) and [^{3}H] or [^{14}C]-labelled fatty acids were purchased from Amersham; silica gel TLC plates (silica K6F) were from Whatman; pyruvate kinase, myokinase and Pefabloc SC were from Boehringer. Acyl-CoA binding protein from *Brassica napus* was expressed in *E. coli* and purified as previously described (Brown et al., 1998). All other chemicals and reagents were purchased from Sigma.

4.2. Enzyme sources

Avocado (*Persea americana*) was purchased locally and stored at 37 °C for 24 hours before use. *E. coli* JM83 and *S. cerevisiae* Y187 were lab stocks and *Candida* 107 was from the Unilever stock centre, Colworth. Mid-development seeds (containing oil) of *Limnanthes douglasii* and sunflower (*Helianthus annuus*) were obtained from greenhouse-grown plants. Oil palm (*Elaeis guineensis*) kernel—12 weeks-post anthesis and developing Cocoa (*Theobroma cacao*), Shea (*Butyrospermum parkii*) and Simarouba (*Simarouba glauca*) seeds were supplied by Unilever. Developing rapeseed (*Brassica napus* var. Falcon) was a laboratory resource. Harvested seed material was stored at −80 °C before microsome and fat pad preparation.

4.3. Preparation of microsomes and fat pads

All homogenisation and centrifugation processes were carried out at 4 °C or below. For homogenisation, plant and microbial material was resuspended (approximately 5 ml of buffer per gram of material) in 100 mM HEPES.NaOH, pH 7.5 containing 0.5 M sucrose, 1 mM DTT and 100 μM Pefabloc. Plant material was homogenised by 4×30 s bursts with a Polytron followed by filtration through 4 layers of muslin to remove large debris, whereas microbial material was lysed by two passages through a cell disruptor (Constant Systems). Samples were centrifuged for 30 min at 20,000 *g* and any resulting floating fat layer was removed using a spatula and retained as the 'fat pad'. The supernatant was re-centrifuged for 1 h at 100,000 *g* to collect the 'microsomal fraction'. Both the microsomal and lipid body fractions were resuspended in a minimal volume

of 100 mM HEPES.NaOH, pH 7.5 containing 100 μM Pefabloc. Aliquots were snap frozen in liquid nitrogen and stored at −80 °C. No decrease in LPA-AT activity was observed over a 12-month period at −80 °C. Some of the fat pads were further purified by dispersion in homogenisation buffer using a glass homogeniser and re-centrifugation at 20,000 *g* for 30 min. This process was carried out three times. Protein concentrations were determined using a Biorad Bradford assay kit with bovine serum albumin as a standard.

4.4. Synthesis of radiolabelled substrates

[^{32}P]-glycerol-3-phosphate (G-3-P) was synthesised in 10 ml reactions containing 100 mM HEPES.NaOH pH 7.5, 2 mM glycerol, 0.5 mM $MgSO_4$, 0.25 mM [γ-^{32}P]-ATP (specific activity 1.67 Bq/pmol) and 20 U *E. coli* glycerokinase (EC 2.7.1.30). The reaction was left overnight at 25 °C and then extracted three times with 50 mg activated charcoal to selectively remove ATP. The proportion of remaining radioactivity in G-3-P was calculated after scintillation counting of spots scraped from silica gel TLC plates of the synthesis mixture developed with *n*-BuOH–HOAc–H_2O (5:4:1). R_f of ATP 0.05, G-3-P 0.15. [^{32}P]-labelled LPA substrates were made from the [^{32}P]-G-3-P in 5 ml reactions containing 0.4 mM G-3-P, 1.2 mM acyl-CoA, 50 mM Tris–HCl pH 6.8, 5 mg/ml BSA and a sample of *A. thaliana* plastidial glycerol-3-phosphate acyltransferase (EC 2.3.1.15) described previously (Brown et al., 1995). The reaction was incubated at 25 °C for 1 h before extraction of labelled LPA into $CHCl_3$ (Brown et al., 1995). After solvent removal with N_2, the substrates (specific activity 0.25–0.33 Bq/pmol) were resuspended in DMSO before storage at −20 °C.

^{3}H- and ^{14}C-labelled acyl-CoAs were synthesised using acyl-CoA synthetase (EC 6.2.1.3) from *E. coli* (FadD), which had been over-expressed using the T7 promoter construct pN324 (Black et al., 1992). FadD was partially purified by chromatography on a Hi-load Q column (Pharmacia) with elution using a gradient from 0–2 M NaCl. Column fractions were assayed (Kameda and Nunn, 1981) and pooled active fractions stored at −80 °C. The synthesis mixture contained, in a final volume of 2.5 ml, 100 mM Tris–HCl pH 7.5, 10 mM ATP, 10 mM phosphoenolpyruvate, 10 mM CoA, 10 mM $MgCl_2$, 20 mM KCl, 50 U pyruvate kinase (EC 2.7.1.40), 50 U myokinase (EC 2.7.4.3), 0.7–1.4 mM [^{3}H]- or [^{14}C]-labelled fatty acid and 200 μl (240 μg protein) of FadD solution. The reaction mixture was incubated for 2–3 h at 30 °C and stopped by addition of 200 μl HCl and 1.25 ml of *iso*-PrOH. Free fatty acids were removed by two sequential extractions with 2 ml of petroleum ether and the labelled product extracted from the remaining aqueous phase with 2×2 ml of *n*-BuOH. The synthesis reaction typically went >90% towards completion, with 70–80% of the labelled acyl-CoA being recovered in the *n*-BuOH fraction. The combined *n*-BuOH fractions were reduced to dryness under a stream of nitrogen and the acyl-CoA resuspended to a concentration of 1–2 mM in 10 mM sodium acetate, pH 6.0, containing 2 mM EDTA and 10 mM ascorbate. The acyl-CoAs were stored under argon at −20 °C until use.

4.5. Analysis of substrates

The purity of [^{3}H]- and [^{14}C]-labelled acyl CoAs was assessed by TLC using *n*-BuOH–HOAc–H_2O (5:2:3) followed by autoradiography (^{3}H-labelled samples) or phosphor-imager analysis (^{14}C-labelled samples). The final preparations contained 80–95% of the label as the acyl-CoA, with free fatty acids being the main contaminant. Some degradation of labelled acyl-CoAs was apparent on prolonged storage and this was assessed and corrected for prior to selectivity assays.

The degree of unsaturation of [^{14}C]-18:2 acyl-CoA was assessed after storage for one month. Argentation TLC of an acetylated hydroxamate prepared from the acyl-CoA as described (Rosenfeld et al., 1975) was carried out, with a methyl ester derived from [^{14}C]-18:2 fatty acid as a reference compound. This showed that 94.3% of the label in the 18:2 acyl-CoA stock was in a di-unsaturated acyl group, with a further 2.7% of the label likely to be an unacetylated hydroxamate derivative and still di-unsaturated.

4.6. Assays

4.6.1. Non-competitive

The standard assay contained, in a final volume of 100 μl: 100 mM Tris–HCl pH 9.0, 0.01% Triton X-100, 1 mg/ml BSA, 2 mM EDTA, 10 mM ascorbate, 100 μM [^{32}P]-labelled LPA (1.67–3.33 kBq), 100 μM acyl-CoA and enzyme sample (1–40 μg protein depending on source). The reaction was started by addition of microsomal material and allowed to proceed for 10–20 min at 30 °C. Samples (100 μl) were removed at appropriate time points and added to 2.5 ml $CHCl_3$/MeOH (1:1) in a screw top glass vial. 1 ml 0.2 M H_3PO_4–1 M KCl was added and the mixture was vortexed briefly then centrifuged (2 min at 3000 *g*) to separate phases. The upper (aqueous) phase was discarded and the lower phase (containing LPA and PA) removed under N_2. The remaining residue was dissolved in approximately 50 μl $CHCl_3$ and applied to a silica gel TLC plate. Substrate and product were resolved by development with $CHCl_3$–MeOH–HOAc–H_2O (25:15:4:2) and radioactive compounds visualised and quantified using a phosphor-imager.

4.6.2. Competitive

The standard assay contained, in a final volume of 100 μl: 100 mM Tris–HCl pH 7.4, 0.01% Triton X-100,

1 mg/ml BSA, 2 mM EDTA, 10 mM ascorbate, 100 μM unlabelled LPA, 10 μM [^{3}H]-labelled 18:1-CoA (2 kBq), 10 μM [^{14}C]-labelled 18:2-, 16:0- or 18:0-CoA (2 kBq) and enzyme sample (0.5–20 μg protein depending on source). The assay was conducted as for the non-competitive assay up to phosphor-imager analysis. Following analysis, spots corresponding to PA were scraped into 1 ml methanol and mixed vigorously for 2–5 min to extract the PA. Silica gel was removed by brief centrifugation and the methanol supernatant subjected to scintillation counting using a dual counting program. It was important to remove the silica as its presence resulted in falsely high tritium counts. Sample volumes and incubation times were chosen such that less than 10% of substrate was consumed over the assay period. Assays routinely comprised 3–4 time points.

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