Identification, Purification and Characterization of Monoacylglycerol Acyltransferase from Developing Peanut Cotyledons*

Ajay W. Tumaney, Sunil Shekar and Ram Rajasekharan†

Department of Biochemistry
Indian Institute of Science
Bangalore 560 012, India
Telephone +91-80-3092881
Fax +91-80-3602627
E-mail lipid@biochem.iisc.ernet.in
ABSTRACT

Biosynthesis of diacylglycerols in plants is mainly through the acylation of lysophosphatidic acid in the microsomal membranes. Here we describe the first identification of diacylglycerol biosynthetic activity in the soluble fraction of developing oilseeds. This activity was NaF insensitive and acyl-CoA dependent. Diacylglycerol formation was catalyzed by monoacylglycerol (MAG) acyltransferase (EC 2.3.1.22) that transferred acyl moiety from acyl-CoA to MAG. The enzyme was purified by successive chromatography separations on octyl-Sepharose, blue-Sepharose, Superdex-75 and palmitoyl-CoA-agarose to apparent homogeneity from developing peanut (Arachis hypogaea) cotyledons. This enzyme was purified to 6,608-fold with the final specific activity of 15.86 nmol min⁻¹ mg⁻¹. The purified enzyme was electrophoretically homogeneous and its molecular mass was 43,000 dalton. The purified MAG acyltransferase was specific for MAG and did not utilize any other acyl acceptor such as glycerol, glycerol-3-phosphate, lysophosphatidic acid and lysophosphatidylcholine. The $K_m$ values for 1-palmitoylglycerol and 1-oleoylglycerol were 16.39 and 5.65 μM, respectively. The $K_m$ values for 2-monoacylglycerols were 2- to 4-fold higher than that of corresponding 1-monoacylglycerol. The apparent $K_m$ values for palmitoyl-, stearoyl- and oleoyl-CoAs were 17.54, 25.66 and 9.35 μM, respectively. Fatty acids, phospholipids and sphingosine at low concentrations stimulated the enzyme activity. The identification of MAG acyltransferase in oilseeds suggests the presence of a regulatory link between signal transduction and synthesis of complex lipids in plants.
Biosynthesis of diacylglycerol is shown to occur by the sequential acylation of glycerol-3-phosphate (1-3). The first enzyme in this pathway, G3P\textsuperscript{1} acyltransferase, catalyzes the formation of lysophosphatidic acid (LPA). LPA can also be synthesized by the acylation followed by the reduction of dihydroxyacetone phosphate that are catalyzed by dihydroxyacetone phosphate acyltransferase (4) and the NADPH-dependent acyl-dihydroxyacetone phosphate reductase (5), respectively. LPA is shown to induce a wide range of activities in animal systems (6-8). LPA can be metabolized by dephosphorylation by a soluble LPA phosphatase to form monoacylglycerol (MAG) or acylated to phosphatidic acid (PA) by LPA acyltransferase. LPA phosphatase has been identified (9), purified (10) and cloned (11) from animal system. PA is the precursor for diacylglycerol (DAG) and anionic phospholipids. PA phosphatase catalyzes the dephosphorylation of PA to form DAG, which is the immediate precursor for triacylglycerol, phosphatidylcholine and phosphatidylethanolamine. DAG is also an important signal molecule that activates protein kinase C (12). DAG can also be derived directly from phospholipids by the action of phospholipase C (13).

Alternatively, DAG can be synthesized by the esterification of MAG by acyl-CoA:MAG acyltransferase (EC 2.3.1.22). This enzyme has been proposed to be important for fat absorption in human small intestine (14-16). Another acyltransferase, acyl-CoA independent MAG acyltransferase, has been purified to homogeneity from rat intestinal mucosa (17) whereas, acyl-CoA dependent MAG acyltransferase has not been purified from any source. All the acyltransferases in these pathways are membrane-bound and most of them use acyl-CoA as a primary acyl donor (1-3).

We identified DAG biosynthetic activity from the soluble fraction of developing peanut (Arachis hypogaea) cotyledons and the enzyme involved was purified to apparent homogeneity by successive column chromatographic procedures and characterized. This is the first report of purification of acyl-CoA dependent MAG acyltransferase.
EXPERIMENTAL PROCEDURES

Materials - [1-14C]Palmitoyl-CoA (54 mCi mmol⁻¹), [9,10-3H(N)]trioleoylglycerol (10 Ci mmol⁻¹), [glycerol-U-14C]PA (100 mCi mmol⁻¹), [[2-palmitoyl-9,10-3H]phosphatidylcholine (92.3 Ci mmol⁻¹) and [2-3H]G3P (12 Ci mmol⁻¹), were obtained from New England Nuclear. Prep grade Superdex 75 (26/60) and Superdex 200 (10/30) FPLC columns, octyl-Sepharose 4 Fast Flow matrix and gel filtration molecular mass standards were purchased from Pharmacia Biotech. Protein assay reagents were obtained from Pierce. Thin layer chromatography plates were from Merck. All other reagents were obtained from Sigma. MAG was purified by preparative silica-TLC and quantified colorimetrically (18, 19). Field grown developing peanut (Arachis hypogaea L.) cotyledons were harvested at 20-25 days after flowering and used either fresh or stored at -80°C until further use.

Lipid extraction - Lipids were extracted from 10 g of frozen immature seeds by grinding the tissue in liquid nitrogen to a fine powder in mortar and pestle (20). The powder was extracted with 20 ml of boiling isopropanol. The mixture was then centrifuged briefly, supernatant removed and the extraction repeated twice. The pooled isopropanol extracts were brought to dryness on a rotary evaporator. The tissue residue was then reextracted twice with 38 ml of chloroform:methanol:10% acetic acid (1:2:0.8, v/v). After centrifugation, the supernatant was added to the isopropanol extract and 20 ml each of chloroform and water were added to the mixture. The biphasic system was mixed and centrifuged. The lower chloroform phase was removed and dried in a rotary evaporator. The lipid residue was dissolved in chloroform:methanol (1:1, v/v) and stored at –20°C.

Preparation of subcellular fractions - Differential centrifugation was used to fractionate intracellular components (21). Either fresh or frozen immature seeds (100 g) were ground in a pre-chilled mortar and pestle with 10 g acid-washed sand and 250 ml buffer consisting of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM KCl, 1 mM MgCl₂, 1
mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg ml⁻¹ leupeptin and 0.25 M sucrose. The extract was passed through two layers of cheesecloth and centrifuged at 3,000 x g for 10 min. The supernatant was centrifuged at 18,000 x g for 15 min. The 18,000 x g supernatant was further centrifuged at 150,000 x g for 2.5 h. The pellets were resuspended in small volume of buffer containing 20 mM Tris-HCl (pH 7.0) and 1 mM β-mercaptopetoethanol. All these operations were performed at 4°C. All the fractions were assayed for acyltransferase activities. Protein concentrations were determined by the bicinchoninic acid method (22) using bovine serum albumin as the standard. For the purification of MAG acyltransferase, the homogenate was centrifuged directly at 18,000 x g for 30 min and the supernatant was then centrifuged at 150,000 x g for 2.5 h. The 150,000 x g supernatant (soluble fraction) was used as the source for MAG acyltransferase.

**Enzyme assays** - The assay mixtures consisted of 50 mM Tris-HCl (pH 7.0), 20 μM [1-14C]palmitoyl-CoA (100,000 dpm), 15 to 45 μg enzyme and 50 μM MAG (1-oleoyl) in a total volume of 100 μl. The incubation was carried out at 30°C for 10 min and stopped by the addition of 400 μl of CHCl₃:CH₃OH (1:2, v/v). Following lipid extraction by the modified method of Bligh and Dyer (23), the lower chloroform-soluble materials were separated by TLC on 250 μm silica gel G plates either using petroleum ether:diethyl ether:acetic acid (70:30:1, v/v) or chloroform:methanol:water (98:2:0.5, v/v) as the solvent system (24). The lipids were visualized with iodine vapor and the spots of DAG scraped off for determination of radioactivity by liquid scintillation counting.

**Purification of MAG acyltransferase** - All operations were conducted at 4°C except FPLC purification step, which was conducted at ambient temperature. Buffer A contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, 1 mM MgCl₂, 1 mM β-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride.
Octyl-Sepharose chromatography - Solid ammonium sulfate was added to bring the soluble fraction to 1 M followed by centrifugation to make the solution clear and then loaded onto an octyl-Sepharose column (4.4 x 15 cm) that had been pre-equilibrated with 1 M ammonium sulfate in Buffer A with a flow rate of 1 ml min⁻¹. The column was washed with the same buffer to remove unbound proteins until the effluent had very low absorbency at 280 nm. The enzyme was eluted with 200 ml of a linear reversed gradient from 1 to 0 M ammonium sulfate in Buffer A and fractions of 5 ml were collected. The active fractions were pooled and dialyzed against Buffer A.

Blue-Sepharose chromatography - Active fractions from the octyl-Sepharose were combined, dialyzed against Buffer A, and applied onto a blue-Sepharose (cibacron blue A) column. The column was eluted with a 0-1 M NaCl gradient.

Size exclusion chromatography - The fractions eluted at 0.35-0.4 M NaCl from the blue-Sepharose were concentrated using a Centricon (30 kDa cut-off) concentrator and filtered. The filtrate was applied onto a preparative Superdex 75 FPLC column fitted with Bio-Rad BioLogic low-pressure chromatography system. The column was eluted with the same buffer at a flow rate of 5 ml min⁻¹.

Palmitoyl-CoA-agarose chromatography - A 2.5-ml of palmitoyl-CoA-agarose was preequilibrated with Buffer A at room temperature and the active fractions from the previous column was mixed with matrix for 90 min at 4°C. The mixture was then poured into a column, washed with Buffer A and eluted with Buffer A containing 0.25, 0.5 and 1 M NaCl, respectively. The MAG acyltransferase activity was eluted at 1 M NaCl.
RESULTS

Subcellular distribution of DAG biosynthesis in immature peanuts

The subcellular distribution of DAG biosynthesis in fresh peanut cotyledons was studied by isolating the intracellular components by differential centrifugation. DAG biosynthetic capacity was found low in the membrane fraction because of the presence of active triacylglycerol biosynthesis (data not shown). However, DAG formation was higher in 150,000 x g supernatant as compared to the corresponding microsomal pellet, which is in agreement with the earlier report on developing rapeseed (25). The pattern of distribution remained the same even in the frozen tissue but total DAG biosynthetic activity decreased 21% after freezing and thawing. These results suggested that the additional DAG biosynthetic machinery could exist in the soluble fraction.

Marker enzyme activities from peanut soluble fraction

There was a significant amount of DAG formation in the soluble fraction and this could either be due to the presence of soluble enzymes or due to the presence of non-sedimentable cellular membrane fragments generated during isolation procedures. Marker enzyme activities were measured to assess the extent of contamination in the soluble fraction with the membranes. The activities of succinate dehydrogenase (26), NADH cytochrome C reductase (27) and vanadium sensitive ATPase (28) were measured as the marker enzymes for mitochondrial, microsomal and plasma membranes, respectively. About 6% (1.2 µmol min⁻¹ mg⁻¹), 18% (1.3 µmol min⁻¹ mg⁻¹) and 11% (0.55 µmol min⁻¹ mg⁻¹) activities of these enzymes were detected in the soluble fraction, respectively. These results indicated that the membrane contamination in the soluble fraction was not significant.

Incorporation of [¹⁴C]palmitoyl-CoA into diacylglycerol

Effect of [¹⁴C]palmitoyl-CoA incorporation into DAG was measured both in the soluble and the membrane fractions of immature peanuts (Fig. 1). Maximum activity was
observed at 10 µM in the soluble fraction and at 20 µM palmitoyl-CoA in the membrane fraction. Exogenous MAG did not alter the rate and the pattern of incorporation into DAG. To determine the amounts of MAG in the immature peanut seeds, the total lipid was extracted as described in Experimental Procedures. The amounts of MAG in the immature seeds and the soluble fraction were calculated to be about 134 nmol g fresh weight\(^{-1}\) and 2.6 to 3.1 nmol mg protein\(^{-1}\), respectively.

**Formation of DAG by the acylation of MAG**

DAG can be synthesized either by the dephosphorylation of PA or by the direct acylation of MAG. To find out the contribution of each step to the total DAG pool, peanut soluble fraction was treated with various concentrations of NaF to inhibit phosphatase activity and these were used for the incorporation of \[^{14}\text{C}\]palmitoyl-CoA into DAG. Initially, we studied the effect of various concentrations of NaF on the dephosphorylation of PA in the membrane fraction and found that there was no formation of DAG at 20 mM NaF (Fig. 2A). As shown in Figure 2B, there was only 25 to 38% decrease in the incorporation of \[^{14}\text{C}\]palmitoyl-CoA into DAG in the soluble fraction in the presence of 20 mM NaF suggesting that the DAG formation was independent of PA dephosphorylation activity. NaF treated membrane fraction showed a profound inhibition of DAG formation indicating the presence of NaF sensitive PA dephosphorylation activity (Fig. 2C). These results suggest that the soluble fraction has NaF-insensitive and PA dephosphorylation-independent DAG biosynthesizing activity in the soluble fraction of immature peanuts.

**Evaluation of different plant tissues for MAG acyltransferase activity**

Incorporation of \[^{14}\text{C}\]palmitoyl-CoA into DAG was studied in the soluble and the particulate fractions from the tissues of leaf and hypocotyl of peanut and immature seed and leaf tissue of castor (*Ricinus communis* L.). The MAG acyltransferase activity was not detected in the soluble fractions of leaf and hypocotyl but the enzyme activity
was in the membrane fractions (data not shown). In peanut leaf, the activity was approximately 48-fold lower than that of immature seed. These data suggested that MAG acyltransferase was also present in other plant tissues but at the low levels.

**Purification of MAG acyltransferase from peanut cotyledons**

MAG acyltransferase activity was found high in the soluble fraction and the rate of synthesis of diacylglycerol in the soluble fraction was 24 pmol min⁻¹ mg⁻¹. Solid ammonium sulfate was added to bring the soluble fraction to 1 M and then loaded onto an octyl-Sepharose column. The column was eluted with a 1 to 0 M linear reversed gradient of ammonium sulfate (Fig. 3A). This step was the most effective that resulted in 221-fold purification of acyltransferase and yielded a 2.6-fold increase in the total activity. The active fractions from the octyl-Sepharose were loaded onto a blue-Sepharose column and eluted with a linear NaCl gradient. The activity was eluted between 0.35 and 0.4 M NaCl (Fig. 3B). The recovery of MAG acyltransferase activity from blue-Sepharose column was nearly 77% of that applied. The pooled active fractions were applied to a preparative Superdex 75 column. The MAG acyltransferase activity was eluted as a single peak from fraction 27 to 31 (Fig. 3C). The active fractions were pooled and applied to a palmitoyl-CoA agarose column as the final step. An overall purification of approximately 6,608-fold was obtained and the specific activity of acyltransferase was 15.86 nmol min⁻¹ mg⁻¹ (Table I). The purified enzyme was resolved on a 12% SDS-PAGE that showed a single band with a molecular mass of 43 kDa (Fig. 4). The native molecular mass of the purified enzyme was found to be 43 kDa by Superdex 200 column chromatography (data not shown).

To confirm that the 43 kDa polypeptide corresponds to the MAG acyltransferase, the purified enzyme was loaded onto a 12% SDS polyacrylamide gel in the presence of 0.1% SDS without boiling and electrophoresed at 4°C. The gel was cut into 0.5-cm sections, the protein eluted from the gel with Buffer A and assayed for the enzyme activity (29). The yield of MAG acyltransferase activity from the gel was low (1.5%) and
the activity was associated with the area of the gel corresponding to the 43 kDa protein (data not shown). These results indicate that the 43 kDa protein detected on the silver-stained gel (Fig. 4) was indeed the MAG acyltransferase.

The reaction products formed at each step of purification were analyzed on a silica-TLC and autoradiographed (Fig. 5). When the soluble fraction was incubated with [14C]palmitoyl-CoA in the presence of 1-MAG (16:0), formation of 1-acyl-, 1,2-diacyl-, and 1,3-diacyl- and triacylglycerols was observed suggesting the presence of many different acylation activities. The active fractions eluted from the octyl-Sepharose column showed very less of other acylation activities which diminished in further purification steps as shown in Figure 5.

**Characteristics of peanut MAG acyltransferase**

The enzyme activity was linear with respect to time and protein concentrations and the pH optimum of the MAG acyltransferase was found to be 7.0. The enzyme was specific for MAG and did not utilize any other acyl acceptor such as glycerol, G3P, LPA and lysophosphatidylcholine. Effect of various detergents on the MAG acyltransferase activity was studied (data not shown). In the presence of 0.3% of Triton X-100 the enzyme activity was reduced to 58%. At 20 mM concentration of the zwitterionic detergent (CHAPS), the enzyme lost its activity completely. The activity of MAG acyltransferase was reduced to 50% in the presence of 40 mM octylglucoside (data not shown).

**Substrate dependencies of MAG acyltransferase**

The substrate specificity of MAG acyltransferase was studied by providing monoacylglycerol of varying chain lengths and position of fatty acid as the substrate. The MAG acyltransferase activity was highest for 1-MAG (16:0) and relatively lower for 2-MAG (16:0). The initial rate of reaction was high for 1-MAG (18:1) but the $V_{\text{max}}$ was less as compared to MAG containing saturated acyl chains. The rate of reaction declined sharply after 10 $\mu$M of 1-MAG (18:1). The 1-MAG (16:0) and 1-MAG (18:1) had
higher $V_{\text{max}}$ values and the $K_m$ values were 16.39 and 5.65 µM, respectively. The other monoacylglycerols had lower $V_{\text{max}}$ and apparent $K_m$ values (Fig. 6A). These results suggested that the MAG acyltransferase preferentially used sn-1-monoacylglycerols.

MAG acyltransferase activity was the highest for palmitoyl-CoA when compared to stearoyl-, and oleoyl-CoAs, but the initial rate of reaction was higher for oleoyl-CoA. The apparent $K_m$ values for palmitoyl-CoA, oleoyl-CoA and stearoyl-CoA were 17.54, 9.35 and 25.64 µM, respectively (Fig. 6B). Competition studies with myristoyl-CoA and lauroyl-CoA showed that the medium-chain acyl-CoAs and acetyl-CoA were not good substrates for the MAG acyltransferase (data not shown). Based on the Lineweaver-Burk plots, apparent $V_{\text{max}}$ and $K_m$ values for acyl acceptors and acyl donors at the standard assay conditions were obtained as summarized in Table II.

**Effect of fatty acids, phospholipids and sphingoid bases on MAG acyltransferase activity**

Effect of fatty acids on the MAG acyltransferase activity was studied using C8 to C18 and C18:1 (Fig 7A). Oleic acid stimulated the MAG acyltransferase activity at concentrations below 15 µM but at the higher concentrations, the activity was reduced. Palmitic acid at concentrations between 20 to 50 µM had a stimulating effect on the enzyme activity and other fatty acids had no significant effect on the MAG acyltransferase activity. Phosphatidylcholine and phosphatidic acid had stimulating effect on the purified MAG acyltransferase activity at lower concentrations from 2.5 to 15 µM and at higher concentrations no stimulatory effect was observed (Fig. 7B). Phosphatidylethanolamine and phosphatidylinositol had no effect on the enzyme activity. At lower concentrations of 2.5 to 7.5 µM, sphingosine and sphingomyelin activated the acyltransferase activity (Fig. 7C). The derivatives of sphingosine, dehydrosphingosine showed lower stimulating effect as compared to sphingosine. Dehydrosphingosine had no effect on the MAG acyltransferase activity.
DISCUSSION
The biosynthesis of diacylglycerol is known to occur via Kennedy pathway in the microsomal membranes (1-3, 30). The present study deals with the first identification, purification and characterization of a soluble MAG acyltransferase from oilseeds. The soluble DAG biosynthesizing activity was observed in immature peanut cotyledons. The presence of a few soluble enzymes that provide precursors for lipid biosynthesis have been reported. A soluble G3P acyltransferase has been isolated from cocoa seed (31). PA phosphatase is found to be localized both in the soluble and the membrane fractions of S. cerevisiae (32, 33) and higher plants (34). A soluble DAG biosynthetic activity has been demonstrated in developing rapeseed (25). LPA phosphatase (9, 10), DAG kinase (35, 36), inactive choline cytidyltransferase (37) and active ethanolaminephosphate cytidyltransferase (38) have also been found in the cytosol of animal systems.

The incubation of NaF-treated soluble fraction with [14C]PA did not generate DAG, however, about 62-75% of [14C]palmitoyl-CoA incorporation into diacylglycerol was observed in the immature peanuts. The synthesized DAG did not originate from the hydrolysis of either triacylglycerol or phosphatidylcholine. These results provide evidences for an alternate enzymatic step for the synthesis of DAG. In this study, we show that DAG is synthesized by the acylation of MAG by acyl-CoA dependent acyltransferase and this enzyme has not been purified from any source. The following observations revealed that the MAG acyltransferase is present in the soluble fraction. i) The activity is associated with 150,000 x g supernatant. ii) The enzyme is permeable in the gel filtration column. iii) The MAG acyltransferase is purified to homogeneity by successive column chromatographic separations without detergent. The role of this enzyme in intracellular processes and its regulation has yet to be elucidated. It appears that different tissues express isozymes with respect to subcellular location. For example, leaf enzyme is found in the particulate fraction but the soluble is found in immature seeds.
We have purified to apparent homogeneity a MAG acyltransferase activity from developing peanut cotyledons by successive chromatographic procedures. The key to the successful purification was the initial step of octyl-Sepharose column chromatography and this step gave a 221-fold purification with a 2.6-fold increase in the total activity. The increase in total enzyme activity could be attributed to the elimination of an inhibitor or the enzymes competing for palmitoyl-CoA. The remaining purification steps showed a successive increase in the specific activity and the fold purification.

The purified peanut MAG acyltransferase showed the highest activity with palmitoyl-CoA but oleoyl-CoA had lower $K_m$ when compared to palmitoyl- or stearoyl-CoAs. Characterization of the partially purified MAG acyltransferase from rat liver (14), intestinal mucosa (39) and adipocytes (40) has also showed higher activity with palmitoyl-CoA. Unlike rat MAG acyltransferase (14), peanut enzyme showed the preference to $sn$-1-monoacylglycerol, over $sn$-2 isomer. This observation could also be attributed to the possible acyl migration from $sn$-2 to $sn$-1 of MAG during conditions of storage, assay or extraction (41). However, we are not certain about either of the two possibilities. Kinetic experiments showed that the overall catalytic efficiencies ($V_{max}/k_m$) for 1-acyl acceptor was higher than that of 2-acyl acceptor suggesting 1-acyl acceptor was a good substrate. The analysis of acyl donors showed that the catalytic efficiency for oleoyl-CoA and palmitoyl-CoA were comparable.

The activity of most of the lipid biosynthetic enzymes is dependent on, or modulated by the various lipid cofactors. In developing oilseeds, lipid biosynthesis is highly active and various metabolic intermediates are accumulated during seed development. All these intermediates either stimulate or inhibit the enzymes involved in lipid metabolism. The characterization of the purified peanut MAG acyltransferase indicated that the lower concentrations of phospholipids and oleic acid stimulated the activity. Apart from oleic acid, palmitic acid also showed activation effect on peanut MAG acyltransferase. On the contrary to our results, it was shown in partially purified
hepatic MAG acyltransferase that the higher concentrations of fatty acid inhibited the enzyme activity (15). Sphingosine was shown to inhibit rat hepatic MAG acyltransferase (42), but the peanut enzyme was activated in the presence of lower concentrations of sphingosine and no inhibition was observed at higher concentrations.

Identification of MAG acyltransferase in peanut indicates the presence of the MAG pathway for DAG biosynthesis. It has been proposed in animal systems that the MAG pathway may play an important role in the regulation of lipid metabolism by controlling the chain-length of fatty acids (43) or controlling the intracellular concentrations of acyl-CoA esters (44) or facilitating selective retention of essential fatty acids during hepatic oxidation (13). In plants, the MAG pathway may be involved in the synthesis of triacylglycerol and may also provide a regulatory link between signal transduction and synthesis of complex lipids. Another possibility is that the MAG pathway contributes to a separate intracellular pool of DAG for different sets of metabolic reaction (45). The identification of the MAG acyltransferase has significant implications in understanding the regulation of di-, and triacylglycerols biosynthesis in plants.

Acknowledgments - We thank Dr. P. N. Rangarajan for helpful discussion and for allowing us to use the FPLC facility. We are grateful to Dr. Savithramma, University of Agricultural Sciences, Bangalore for giving us peanut and castor seeds.
REFERENCES


FOOTNOTES

*This research was supported by Department of Science and Technology, and Department of Biotechnology, New Delhi, India.

✝To whom correspondence should be addressed.

Ram Rajasekharan, Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India.

Telephone: +91-80-3092881; Fax: +91-80-3602627

E-mail: lipid@biochem.iisc.ernet.in

†The abbreviations used are: DAG, diacylglycerol; FFA, free fatty acid; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; TAG, triacylglycerol.
FIGURE LEGENDS

Fig. 1. Effect of palmitoyl-CoA on diacylglycerol biosynthesis. Incorporation of [14C]palmitoyl-CoA into DAG was carried out in the presence of 50 µM MAG in the (○) soluble and the (●) membrane fractions. Each point is the average of two independent experiments.

Fig. 2. Generation of diacylglycerol. (A) Effect of NaF on the dephosphorylation of [14C]PA to DAG was determined in the soluble (○) and the membrane (●) fractions. The incorporation of [14C]palmitoyl-CoA in the presence (○) or in the absence of (●) of 20 mM NaF was performed with (B) the soluble and (C) the membrane fractions into DAG in the absence of exogenous acyl acceptor. Each point is an average of three determinations.

Fig. 3. Elution profile of MAG acyltransferase on various column chromatography. (A) The soluble fraction from immature peanuts was loaded onto an octyl-Sepharose column that was preequilibrated with 1 M ammonium sulfate. The MAG acyltransferase was eluted from the octyl-Sepharose column in a reserved linear gradient of ammonium sulfate (---) and 5 ml fractions were collected. (B) The active fractions from the octyl-Sepharose were pooled and loaded onto a blue-Sepharose column. The MAG acyltransferase was eluted in a linear gradient of NaCl (---) and 1.5 ml fractions were collected. (c) Active fractions from the blue-Sepharose were pooled and loaded onto a preparative gel filtration column (Superdex 75), and 5-ml fractions were collected. All the fractions from various column were assayed for the MAG acyltransferase activity (○) and the protein (●).
Fig. 4. SDS-PAGE profile of MAG acyltransferase purification. Samples from each purification step were separated on a 12% SDS-PAGE. Lanes 1-5 correspond to the pooled fractions from the steps 1-5 (Table I). Lane Mw represents the standard molecular weight marker.

Fig. 5. Autoradiography of TLC profile of the reaction products formed at each step of purification. The enzyme was assayed using \[^{14}\text{C}]\text{palmitoyl-CoA}\) and 1-MAG (16:0), and the products formed were chromatographed using (A) petroleum ether:diethyl ether:acetic acid (70:30:1, v/v) and (B) chloroform:methanol:acetic acid (98:2:0.5, v/v) as the solvent systems. Lane 1 represents heat inactivated soluble fraction; Lanes 2-6 correspond to the active fractions from steps 1-5 (Table I).

Fig. 6. Lineweaver-Burk plot of MAG acyltransferase activity towards monoacylglycerols and acyl-CoAs. (A) The enzyme activity was measured as the function of MAG concentrations and the concentration of palmitoyl-CoA (20 \(\mu\text{M}\)) was kept constant. (B) The enzyme activity was measured as the function of acyl-CoA concentrations and 1-palmitoyl-\(\text{sn}\)-glycerol (20 \(\mu\text{M}\)) was kept constant. Each point is the average of two determinations.

Fig. 7. Effect of fatty acids, phospholipids and sphingoid bases on MAG acyltransferase activity. The MAG acyltransferase activity was measured under the standard assay conditions using \[^{14}\text{C}]\text{palmitoyl-CoA}\) and 1-palmitoyl-\(\text{sn}\)-glycerol in the presence of various concentrations of (A) fatty acids, (B) phospholipids and (C) sphingoid bases. Each point is the average of two determinations.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total activity (nmol/min)</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>1721.6</td>
<td>4.13</td>
<td>0.0024</td>
<td>1</td>
</tr>
<tr>
<td>Octyl-Sepharose</td>
<td>20.15</td>
<td>10.68</td>
<td>0.53</td>
<td>221</td>
</tr>
<tr>
<td>Blue-Sepharose</td>
<td>1.56</td>
<td>8.17</td>
<td>5.24</td>
<td>2,183</td>
</tr>
<tr>
<td>Superdex 75</td>
<td>0.45</td>
<td>5.50</td>
<td>12.22</td>
<td>5,092</td>
</tr>
<tr>
<td>Palmitoyl-CoA-agarose</td>
<td>0.10</td>
<td>1.59</td>
<td>15.86</td>
<td>6,608</td>
</tr>
</tbody>
</table>

The results are the summary of purification of the MAG acyltransferase. Frozen immature seed (100 g) was used for preparing the soluble fraction. The enzyme activity measurement and the purification steps are described in Experimental Procedures.
TABLE II

Kinetic parameters of MAG acyltransferase

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-MAG (16:0)</td>
<td>16.39</td>
<td>16.9</td>
<td>1.03</td>
</tr>
<tr>
<td>2-MAG (16:0)</td>
<td>30.30</td>
<td>13.4</td>
<td>0.44</td>
</tr>
<tr>
<td>1-MAG (18:1)</td>
<td>5.65</td>
<td>15.8</td>
<td>2.80</td>
</tr>
<tr>
<td>2-MAG (18:1)</td>
<td>22.73</td>
<td>13.7</td>
<td>0.60</td>
</tr>
<tr>
<td>1-MAG (12:0)</td>
<td>58.81</td>
<td>11.1</td>
<td>0.19</td>
</tr>
<tr>
<td>16:0-CoA</td>
<td>17.54</td>
<td>20.0</td>
<td>1.14</td>
</tr>
<tr>
<td>18:0-CoA</td>
<td>9.35</td>
<td>17.9</td>
<td>1.91</td>
</tr>
<tr>
<td>18:1-CoA</td>
<td>25.64</td>
<td>9.4</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Apparent $K_m$ and $V_{max}$ values were calculated from data in Fig. 6.
Fig. 1

Tumaney et al.

![Graph showing the formation of DAG (pmol/min/mg) with increasing concentration of 16:0-CoA (µM). The graph has two lines: one with circles and one with dots, indicating different conditions or treatments.]
Fig. 4

Tumaney et. al.

43 kDa

Mw 1 2 3 4 5

14.3 29 43 66 97.4 kDa
Fig. 5

A

Tumaney et. al.

B

1 2 3 4 5 6
Fig. 6  Tumaney et al.

A

B
Fig. 7

A

DAG formed (nmol/min/mg) vs Fatty acids (µM)

- C 8:0
- C 12:0
- C 14:0
- C 16:0
- C 18:1
- C 18:0

B

DAG formed/min/mg vs Phospholipids (µM)

- PI
- PA
- PE
- PC

C

DAG formed (nmol/min/mg) vs Lipids (µM)

- Sphingosine
- Dihydrosphingosine
- Hydroxy-sphingosine
- Sphingomyelin
Identification, purification and characterization of monoacylglycerol acyltransferase from developing peanut cotyledons
Ajay W. Tumaney, Sunil Shekar and Ram Rajasekharan

J. Biol. Chem. published online January 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100005200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts