Biosynthesis of α-elostearic Acid in the Seed of Momordica Charantia L.

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Research Article

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Abstract

Momordica charantia L. is a plant belonging to *Cucurbitaceae* family. Currently it is cultivated throughout the word mostly for the immature fruits. Its seeds oil contains a large amount of α-eleostearic acid (αESA) , an isoform of α-linolenic acid with conjugated double bound. Oils with conjugated fatty acids are valuable both for industrial and nutraceutical application. After cloning the fatty acid conjugases (FADX), several attempts have been made to modify oilseed crops towards production of such fatty acids. The obtained transgenic plants produced, however, a much lower amount of conjugated fatty acids than *FADX* original plants. It has been postulated that this could be connected with the problem in the transfer of such fatty acids from the place of its synthesis – phosphatidylcholine (PC) – to the place of their storage – triacylglycerol (TAG) in the transgenic plants. In this study we have characterised the biosynthesis of α-eleostearic acid both *in vivo* in developing seeds of *M. charantia* and *in vitro* in experiments with microsomal fractions prepared from developing seeds of this plant. We observed significant differences in transfer of αESA from the place of its biosynthesis to TAG in these two system. *In vivo* αESA was very efficiently transferred while *in vitro* synthesised αESA remained mostly in PC.

Introduction

Momordica charantia L (bitter melon or karela) is a plant native to eastern India and southern China [1]. Bitter melon is adapted to a wide variation of climates and now is cultivated throughout the word mostly for the immature fruits [2]. The seeds of bitter melon contain 33-36% of oil [3, 4], however, even as high value as 47.5% of oil content in its seed was reported [5]. The bitter melon oil can be utilised for human consumption after proper refining [4], however, due to the high contents of fatty acids with conjugated double bound (drying agent) it is commercially used for coating materials and inks [4, 6]. Conjugated fatty acids are isoforms of α-linolenic acid in which adjacent double bonds are not separated by a methylene group. One of such fatty acids is present in bitter melon oil: α-eleostearic acid (cis-9,trans-11,trans-13-octadecatrienic acid). According to different sources it constitutes 50-53% [4], about 60% [3] or even 65% [7] of its fatty acids. Besides bitter melon, α-eleostearic acid is present in large amounts in seeds oil of *Alurites fordii* where is constitutes 77 to 86% of the fatty acids of its oil [8]. Conjugated fatty acids are also present in a limited number of other plant species. In *Catalpa ovate* exists, for instance, catalpic acids (trans -9,trans-11,cis-13-octadecatrienoic acid). In *Jacauranda mimosifolia* jacaric acid (cis-8,trans-10,cis-12-octadecatrienoic acid), in *Calendula officinalis* calendulic acid (trans-8,trans-10,cis-12-octadecatrienoic acid) and in *Punica granatum* punic acid (cis-9,trans-11,cis-13-octadecatrienoic acid), [9].

In the past, different mechanisms have been proposed to explain the formation of conjugated fatty acids. Including that they can be formed *via* isomerisation of α-linolenic acid, or *via* formation of linolenic acid radicals in lipoxygenase-type of reaction or *via* a formation of epoxy derivatives of linoleic acid [3]. Using different radioactive precursors they obtained evidence that linoleate (18:2) is the acyl precursor of α-eleostearic acid (αESA) and that its conversion to αESA occurs while 18:2 is esterified to phosphatidylcholine (PC). Letter on it has been demonstrated that the conversion of 18:2 to conjugated

trienoic-acids is done by divergent forms of Δ12 desaturase, which has been designed as 'fatty acid conjugases or FADX' [7, 10, 11].

Introduction of fatty acid conjugases encoding gene to other oilseed plants like *Arabidopsis thaliana* [6, 11], *Brassica napus* [12] or soybean [6, 7] resulted, however, in much lower levels of these types of fatty acids in the seeds of transgenic plants compared to FADX native plants. Combining transformations of *FADX* and *FAD2* desaturase from plants natively accumulating conjugated fatty acid seems to provide some help in increasing the amount of conjugated fatty acids in transgenic plants. Mietkiewska et al. [13] showed that combined transformation of *A. thaliana* with *FADX* and *FAD2* desaturase from *P. granatum* increased the accumulation of punicic acid up to 21% of total fatty acids of *Arabidopsis* seeds compared with 4.4% obtained previously when only *FADX* from *P. granatum* was introduced [11, 13]. However, this was still a much lower amount than up to 80% of punicic acid in oils of *P. granatum*. Thus, this indicates that additional genes/enzymes connected with transfer of conjugated fatty acids from place of its synthesis – PC - to triacylglycerols has to be first identified and then expressed together with *FADX* to obtain transgenic plants producing high amount of these fatty acids.

The conjugated fatty acids similarly to other products of desaturases e.g. polyunsaturated fatty acids or fatty acids with hydroxy or epoxy group, could be transferred from PC (place of its biosynthesis) to the cytosolic pool of acyl-CoA available for TAG synthesis e.g. *via* the backward reaction of acyl-CoA:lysophoshatidylcholine acyltransferases (LPCATs), [14, 15]. Fatty acids modified in PC could also enter TAG as diacylglycerols (DAG) with *de novo* synthesised polyunsaturated or uncommon fatty acids. Such DAG molecules can be provided by the action of CDP-choline:diacylglycerol cholinephosphotransferase (CPT), or phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) [16, 17]. The fatty acid modified in PC can be also directly transferred to diacylglycerols producing TAG via the action of phosholipid:diacylglycerol acyltransferases (PDAT), [18, 19]. The phospholipase C and phospholipase A2 can be also involved [6]. So far, however, the relative contribution of the enzymes potentially involved in the transfer of conjugated fatty acids from PC to TAG has not been characterised at all.

In the presented study we characterised the biosynthesis of α-eleostearic acid in developing seeds of *M. charantia* L. The experiments were divided in 2 parts. The first concerned the occurrence and accumulation of αESA *in vivo* in developing seeds of *M. charantia*. The second one included *in vitro* experiments with microsomal fractions prepared from developing seeds of this plant. We observed considerable differences in transfer of αESA from the place of its biosynthesis – PC – to TAG in these two system. *In vivo* αESA was very efficiently transferred while *in vitro* synthesised αESA remained mostly in PC, similarly to transgenic plants carrying the gene of FADX [6].

Results

Lipid accumulation in developing seeds of *Momordica charantia*

The analyses were performed at four stages of seeds development: 20 DAP (days after pollination), 23 DAP, 26 DAP and 33 DAP. At 20 DAP the seeds contained only about 3.5% of lipids (measured as the amount of fatty acids in acyl lipids/seed) present in seeds at 33 DAP. During next 3 days of development the lipid contents in the seeds increased to about 28.5% of its final amount in mature seeds (33 DAP). In the following three days lipid accumulation was the most intensive and at 26 DAP reached almost 64% of its final amount. During the final 6 days, the lipid accumulation slowed down, however, at that time seeds accumulated the remaining 36% of lipids (Fig. 1 and Table S1). The main lipid classes were triacylglycerols (TAG). Already at 20 DAP they accounted for about 75% of all acyl-lipids and their relative amount reached about 98% at 33 DAP. Polar lipids (all phospho- and glyco-lipids measured as one class) and diacylglycerols (DAG) at 20 DAP accounted for about 21% and 3,6% respectively of all acyl-lipids and its relative amount gradually decreased to about 1.4% and 0.5% (respectively) at 33 DAP. The absolute amount of TAG/seed was increasing contentiously during the seeds development, while polar lipids and DAG reached their maximum level/seed at 26 DAP, with smaller increases between 23 and 26 DAP of 7 and 17% respectively of their maximum value (Fig. 1 and Table S2).

Fatty acids of acyl-lipids of developing seeds of *Momordica charantia* The lipids of *M. charantia* seeds contained five main fatty acids: palmitic acids (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and α-eleostearic acid (αESA). At 20 DAP the relative amount of 16:0 accounted for about 7.9% of all fatty acids and its relative amount gradually decreased to 1.6% in mature seeds. The relative amount of 18:0 was low at the very early stages of seeds development (about 7.7% at 20 DAP) and increased to about 19% in subsequent stages. The relative amount of 18:1 and 18:2 accounted for about 31% and 38% respectively at 20 DAP and decreased to about 10% and 7% (respectively) at 33 DAP. The content of αESA at 20 DAP was close to 12% of all fatty acids of *M. charantia* seeds' lipids and its relative amount increased to about 58% in the mature seeds (Table S1).

The absolute amount of all fatty acids, present in acyl-lipids of developing *M. charantia* seeds, gradually increased during the seeds development. However, the rate of its accumulation differed. The amount of 16:0 and 18:2/seed between 20 DAP and 33 DAP increased about 5.5 times, the amount of 18:1 - about 10 times, the amount of 18:0 - about 72 times and αESA about 141 times (Fig. 2). In case of αESA and 18:0 the highest rate of accumulation occurred between 23 and 26 DAP and accounted for about 8.8 and 2.8 μmol/day/seed respectively. The highest rate of accumulation of 16:0, 18:1 and 18:2 occurred between 20-23 DAP and accounted for about 0.15, 1.23 and 1.09 μmol/day/seed respectively (Table S3).

The fatty acids described above were not equally distributed among different lipid classes of developing *M. charantia* seeds. The αESA was a dominated fatty acid in TAG and DAG except for the first stage of seed development (20 DAP). At that time 18:1 and 18:2 were the dominating fatty acids in these lipids. The content of αESA in TAG increased from about 53% in 23 DAP to about 60% in 33 DAP. In case of DAG αESA constituted already in 23 DAP 37% of its fatty acids and this value increased further to about 44-45% in 26 and 33 DAP. The content of αESA in polar lipids was low, however, it gradually

increased from about 1.5% in 20 DAP to about 6% in 33 DAP. The linoleic acid was the dominated fatty acid in polar lipids at all stages of seeds development. Its relative amount accounted for about 51-70% of all fatty acids and reached the highest levels in 23 and 26 DAP. Except for 20 DAP the relative amount of 18:2 in DAG remained relatively stable at 11-13% of all fatty acids. Its amount in TAG decreased from about 27% in 20 DAP to about 5.7% at 33 DAP. The differences in distribution of 16:0, 18:0 and 18:1 between different lipid classes were less pronounced compared to αESA and 18:2 (Fig. 3).

The ability of microsomal fraction of developing seeds of *Momordica charantia* to synthesise in vitro α-eleostearic acid

Microsomal fractions were prepared from developing seeds at 26 DAP. At that time the seeds showed still high ability for biosynthesis/accumulation of α-eleostearic acid (αESA) and were big enough to easily provide sufficient amount of material for microsomal fraction preparation. In the assays microsomal fractions (aliquots containing 112 nmol microsomal PC; about 612 nmol of FA of all acyl-lipids) were incubated without and with NADH (4mM) for 0, 10 and 30 min. After that time lipids were extracted, chloroform fraction methylated with 0.1 M NaOH in dry methanol and obtained fatty acid methyl esters analysed on GC (for more details see Material and Methods). At 0 min incubation the αESA accounted for about 9% of all fatty acids present in complex lipids of the analysed microsomal fractions. Its relative amount subsequently increased to about 10.5% and 13% after 10 and 30 min incubation (respectively) with NADH. On the contrary, in case of assays without NADH its relative amount did not increase and even small decreases occurred. The observed relative increase of αESA in sample with NADH accounted for about 9.6 and 24.2 nmol (after 10 and 30 min incubation) of real increases of αESA/assays. At the same time, the amount of 18:1 and 18:2 decreased by about the same amount as the increase of αESA in the assays with NADH. The relative changes of 16:0 and 18:0 in assays with NADH and the relative changes of all analysed fatty acids in assays without NADH were small (Table 1).

In vitro biosynthesis of α-eleostearic acid from exogenous substrates by microsomal fractions from developing seeds of *Momordica charantia*

The research were conducted with microsomal fractions prepared from developing seeds of *M. charantia* at 26 DAP as they showed good ability (see p. 3.3.) to synthesise α-eleostearic acid (αESA). In the experiments we used [^{14}C]18:1-CoA, [^{14}C]18:2-CoA and [^{14}C]18:3-CoA as potential exogenous substrates. After incubation of microsomal fractions with these precursors (in the presence of exogenous NADH) lipids were extracted to the chloroform and methylated. The obtained fatty acid methyl esters were then separated on TLC impregnated with $AgNO_3$. This allowed for separation of 18:1-Me, 18:2-Me and 18:3-Me from each other. In the assays with [^{14}C]18:1-CoA two main labelled spots were detected: one co-localised with 18:1-Me (or αESA-Me) and the other co-localised with 18:2-Me. Thus, these assays indicated clearly that Δ12 desaturase was active in the tested microsomal fractions, however, any potential synthesis of αESA could not be detected in the separation system we used. When we incubated the microsomal fractions with [^{14}C]18:2-CoA we saw also two main labelled spots: co-localised with 18:2-Me and co-localised with 18:1-Me/αESA-Me. The radioactivity localised on the last spot accounted for

about half of the radioactivity in the line. Later on we found out that [^{14}C]18:2-CoA we used in the experiment contained about 20% of contamination (probably trans-18:2-CoA) which migrated after methylation together with 18:1-Me/αESA-Me. Nevertheless, still about 30% of added radioactive acyl-CoA was converted to αESA (in the further studies we have purified [^{14}C]18:2 from the contamination before synthesising [^{14}C]18:2-CoA). In the assays with [^{14}C]18:3-CoA, we did not detect any formation of [^{14}C]αESA. Only probably some oxidative products (which migrated faster than 18:3-Me) were formed (Fig. 4). This experiment clearly showed that from amongst the tested substrates only [^{14}C]18:2-CoA can be useful in the subsequent studies of in vitro biosynthesis of αESA.

The biosynthesis of α-eleostearic acid from [^{14}C]18:2-CoA - effect of microsomes amounts and time dependency

For the experiments we used five different microsomal concentrations; aliquots containing 6, 12, 24, 48 and 96 nmol of endogenous PC. The production of [^{14}C]αESA was observed in all assays, however, the amount of formed [^{14}C]αESA was increasing with the increase of microsomal fraction in the assays up to 24 nmol of microsomal PC (between 6 and 12 nmol microsomal PC - in a linear way). Further increases of microsomal fraction in the assay did not result in higher production of [^{14}C]αESA (Fig. 5**a**). For further experiments we decided to use aliquots of microsomal fraction containing 24 microsomal PC as this amount of microsomes gave the highest production of [^{14}C]αESA.

For time dependency experiments the same conditions as in the experiment described above and aliquots of microsomes containing 24 nmol of microsomal PC was used. It was possible to detect a small production of [^{14}C]αESA already after 1 min incubation time (in this time about 66 pmol of [^{14}C]αESA was formed). During the next 9 min incubation the amount of produced [^{14}C]αESA increased about 10 times. The synthesis of [^{14}C]αESA continued up to 60 min of incubation (at that time about 12% of added radioactivity was in [^{14}C]αESA). Subseqnetly no further increases of the amount of produced [^{14}C]αESA occurred (Fig. 5**b**).

In the assays of microsomal fractions without any addition of exogenous precursors of αESA we showed that the biosynthesis of these fatty acids occurs only in the assays containing NADH. To confirm this, we performed the additional time dependency experiments were half of the assays contained NADH (4 mM) and halve did not. The production of [^{14}C]αESA in the assays with NADH was similar to the one presented above. However, there were no signs of [^{14}C]αESA in the assays without NADH (Fig. 6). Thus we confirmed that conversion process of 18:2 to αESA requires a reduction factor like NADH.

Localisation of the radioactivity from exogenous [^{14}C]18:2-CoA and lipids were [^{14}C]α-eleostearic acid was detected

Radioactivity from added [^{14}C]18:2-CoA was found in polar lipid fraction, diacylglycerol fraction (DAG), free fatty acid fraction (FA) and triacylglycerol fraction (TAG). Most of the radioactivity was

detected in polar lipids (up to 94-% of the radioactivity of chloroform fraction). With incubation time the amount of radioactivity in polar lipids decreased whereas in FA, DAG and TAG it increased (Fig. 7).

To verified at which lipid classes [^{14}C]αESA is localised, separated polar lipids, DAG and TAG were scrapped of from the TLC plates and transmethylated with 0.1 NaOH in dry methanol. The FA fractions were eluted from the gel to hexane. Obtained methyl esters and FA were separated on TLC impregnated with $AgNO_3$ and [^{14}C]FA-Me/[14C]FA visualised on the plate in IMAGER. The obtained results, showed that up to 10 min of incubation time [^{14}C]αESA was observed only in the polar lipid fraction (Fig. 8). After 30 and 60 min incubation time the [^{14}C]αESA was found also in DAG, FA and TAG, however its amount was low –in all three fractions together up to 7% of [^{14}C]αESA amount found in polar lipids (data not presented – too low intensity of the [^{14}C]αESA-Me bounds).

To further investigate the localisation of the added radioactivity in different lipid classes we pre-incubated the microsomal fractions (48 nmol microsomal PC/assays; double volume of buffer compared to standard assays condition) with 20 nmol [^{14}C]18:2-CoA without NADH. After pelleting the microsomes (20 min centrifugation at 13,000 rpm) new incubation buffer (100 µl) without [^{14}C]18:2-CoA and with NADH (4 mM) was added and microsomes were incubated again for 0, 10, 30 and 60 min. Aliquots (20%) of chloroform fractions were separated on TLC with polar solvents. At 0 min time almost 86% of radioactivity was localised in PC and with incubation time this amount dropped to about 72% after 60 min. In neutral lipids (TAG, DAG, FA) at 0 min about 5.6% of the radioactivity was localised. After 60 min incubation this amount increased to about 14%. In PE there was about 4.6% at 0 min and about 5.8% after 60 min incubation time. Other polar lipids contained small amount of the radioactivity; usually not exceeding 2% (Fig. 9). Remaining 70% of chloroform fraction (10% was used to measure the radioactivity in chloroform fraction) was separated on TLC with polar solvent (to each sample 50 nmol of di-16:0-PC was added) and lipids were visualised on the plates by spraying with water. Areas containing PC, neutral lipids (TAG, DAG, FA) and remaining parts of chromatogram were scrapped from the plate and methylated with 0.1 M NaOH in dry methanol. Obtained methyl esters were than separated on TLC impregnated with $AgNO_3$. [^{14}C]αESA was found in PC (5.8% of the radioactivity after 10 min and 10.2 % after 60 min incubation) and in neutral lipids (9.1% after 10 min and 14.7% after 60 min incubation). Chloroform fraction from 0 min incubation (directly methylated without prior separation on TLC with polar solvent) contained only trace amount of [^{14}C]αESA (Fig. 10). The methylation in situ of polar lipids from the rest of the chromatogram was not successful; lipids were oxidised during the methylation and localised at the start of the chromatogram (data not presented).

Discussion

Oils with conjugated fatty acids have both industrial application and nutraceutical application as a food with medicinal benefits [13]. After cloning the fatty acid conjugases, several attempts have been made to modify oilseed crops, however, without any spectacular results. The transgenic plants produced conjugated fatty acids in a much lower level than *FADX* origin plants (see Introduction). Thus, it was

suggested that the problem may lie in the transfer mechanism of such fatty acids from their place of synthesis – PC – to the place of storage – TAG [6, 13]. Cahoon et al. [6] considered also the possibility that different plant species accumulating conjugated fatty acids may have different transfer mechanisms. In the presented studies we tried to characterise the biosynthesis and transfer of α-eleostearic acid (αESA) both *in vivo* in developing seeds of *M. charinata* and *in vitro* in assays with microsomal fraction isolated from developing seeds of this plant.

The transfer of αESA from PC to TAG *in vivo* is very efficient; especially in *M. charantia* seeds development between 23 and 26 DAP. The polar lipids content at that time of seeds development amounts to around 0.8 μmol/seed. This means that PC content will amount to around 0.4-0.5 μmol/seed (PC constitutes usually 50-60% of all polar lipids). At that time of seed development as much as about 8.8 μmol of αESA was synthesised and transferred to TAG during each day of seed development. Taking into consideration that αESA is synthesised mostly at sn-2 position of PC all PC molecules have to be remodelled during one day approximately 18-22 times (1.5 – 1.8 times/h); [6]. This is a couple of times more intensive remodelling than the one occurring in *Camelina sativa* seeds [22]. However, in *C. sativa* only the total fatty acid exchange in PC *via* backward reaction of LPLAT was evaluated. Over 90% of sn-2 position of TAG of *M. charantia* mature seeds is occupied by αESA [6]. Thus, we have to considered, that DAG utilised for TAG biosynthesis in this plant has originated from PC. In our studies we have shown that DAG molecules present in the developing seeds of *M. charantia* constitute up to 45% of αESA which is in line with above suggestion. The amount of DAG molecules in the developing seeds of *M. charantia* is relatively low and during the most intensive time of lipid accumulation varies between 0.5-0.6 μmol/seed (from 1.5% - 23DAP - to 0.5% - 33 DAP - of all lipids). This indicates that utilisation of DAG generated (most probably) from PC is very efficient. The majority of those DAG molecules are utilised for TAG biosynthesis *via* the DGAT or PDAT action. So far there are no data about the activity of these enzymes in *M. charantia* seeds, thus we cannot speculate on the relative importance of the mentioned enzymes in the biosynthesis of triacylglycerols. LPCAT type of enzymes are probably the suppliers of αESA-CoA for TAG biosynthesis *via* DGAT action - however there no studies exploring this mechanism in *M. charantia*. Only the transcript for DGAT1, DGAT2, PDAT1, LPCAT, phospholipase C and other enzymes potentially connected with biosynthesis and transfer of αESA were detected in *M. charantia* seeds extracts [23].

To compare the biosynthesis and transfer of αESA in cell-free environment with the one occurring in intact cells, we prepared microsomal fractions from developing seeds of *M. charantia*. In the assays with these fractions we got a fairly good rate of biosynthesis of αESA *in vitro*. Using only endogenous/microsomal substrate we observed in assays with NADH a rate of the *de novo* synthesis of αESA molecules corresponding to 43-51% (depending on incubation time) of total PC molecules per one hour. This is a lower rate than the discussed above formation of αESA in developing seeds of *M. charantia.* However, if we take under consideration the fact that microsomal fraction was prepared from seeds at 26 DAP and that between 26-33 DAP the rate of biosynthesis of αESA was about half of the rate discussed above, the proximate rate of synthesis of αESA molecules *in vivo* during this time could correspond to 75-90% of seeds PC molecule per hour and these values are only about 1.7 times higher than obtained in *in vitro* assays.

So far it has been presented a several evidence that exogenous 18:2 is converted to αESA *in vivo* by endogenous or introduced to yeast system fatty acid conjugases [3, 10, 11]. In the experiments presented here, we have shown that this conversion is also effective *in vitro* in assays with microsomal fraction from developing seeds of *M. charantia*. We have also shown that this conversion occurred under condition that exogenous NADH is added to the assays. We used [^{14}C]18:2-CoA as a source of exogenous 18:2. Added linoleic acid was very rapidly incorporated into microsomal polar lipids (up to 94% of [^{14}C] of chloroform fraction) indicating that a very active LPLAT type of enzymes existed in the used microsomal fractions. As the vast of majority of this radioactivity was concentrated in PC most of these enzymes were probably of LPCAT type. Introduction of linolenic acid derived from [^{14}C]18:2-CoA to PC can take place both *via* forward and backward reaction catalysed by LPCAT [24]. This indicates also indirectly that LPCAT type of enzymes can be involved in the transfer of αESA from the place of its synthesis to storage TAG.

The *in vivo* formed αESA was very rapidly transferred from the place of its biosynthesis to TAG. Polar lipids of developing seeds of *M. charantia* contained only 1.5-2% of this fatty acid at 20 and 23 DAP and its amount increased to about 4.2 and 6.1% at 26 and 33 DAP. The very low amount of αESA in PC of *M. charantia* seeds was also reported earlier [3, 6]. Contrary to *in vivo* situation the vast majority of formed *de novo* [^{14}C]αESA *in vitro* stayed in PC. In assays without pre-incubation only about 7% of *de novo* formed [^{14}C]αESA was transferred to neutral lipids (DAG, TAG, FA) and in assays with pre-incubation about 19% during 1 h incubation time. The situation was a bit similar to that with transgenic plants, where very bad transfer of αESA from PC to TAG was noticed [6]. As the microsomal fraction was derived from the same seeds which *in vivo* expressed a very active transfer of αESA from PC to TAG the most probable explanation could be that some factor involved in this transfer was missing during microsomes preparation. The membrane bound enzymes like conjugase, desaturase FAD2 and LPCAT were very active in the prepared microsomes indicating that also other membrane bound enzymes like DGAT or PDAT could be active. Consequently, this suggests that the critical component/s missing during microsomes preparation could be a soluble one. In *in vitro* assays not only transfer of [^{14}C]αESA to TAG was very low but also [^{14}C]DAG was synthesised at a very slow rate. Thus, the amount of formed DAG could be a limiting factor during this transfer (as DAG is a direct precursor of TAG synthesis). DAG molecules with [^{14}C]αESA can be formed from [^{14}C]PC via CPT or PDCT action [16, 17]. The phospholipase C can be also involved [6]. PDCT probably is not present in *M. charantia* as a transcript of the gene encoding this enzymes was not found in this plant [23]. The CPT is mostly present in ER [25] thus should be also present in the microsomal fraction. However, the localisation of specific for PC phospholipase C (PC-PLC) is not yet defined in spite of genes encoding these enzymes have already been cloned [26]. Thus, we cannot excluded that the missing factor in αESA transfer from PC to TAG in our assays is PC-PLC. This proposition, however, requires further experimental evidence.

Materials And Methods

Chemicals

[1-^{14}C]-labelled fatty acids were purchased from Amersham Biosciences (UK) and non-radioactive fatty acids and non-radioactive lipid standards from Larodan (Malmö, Sweden). Free CoA, bovine serum albumin (BSA), NADH and heptadecanoic acid methyl ester (17:0-Me) were supplied by Sigma-Aldrich (St. Louis, MO, USA). The [1-^{14}C]-labelled acyl-CoAs were prepared according to the modified methods described by Sanchez et al [20]. The other chemicals and solvents used for analysis were from Merck (Darmstadt, Germany) or Sigma-Aldrich.

Plant materials

Analyses were performed on *M. charantia* L. Plants were grown from seeds in growth chamber at 20/24 ^{o}C night/day temperature with 60% relative humidity and with a 14h photoperiod at a light intensity of 120 µmol photons m^{-2} s^{-1}. About 4-5 weeks after planting, plants started flowering. The selected flowers were manually pollinated. The developing fruits were harvested after 20, 23, 26 and 33 days after pollination (DAP) and used for seeds separation. During the first three harvest times, the seeds had white coats and were relatively soft. At 33 DAP the seeds had read coat and were hard. We treated them as mature (or almost mature) seeds. The freshly harvested seeds were used for lipid analyses and for microsomal fractions isolation.

Lipid analyses

Lipids extraction from seeds of *M. charantia* was done according to modified methods described by Bligh and Dyer [21]. Single seed (after removing seed coat) were homogenised in Potter-Elvehjem homogenizer with 3.75 ml of chloroform:methanol (1:2; v:v) with subsequent addition of 1.25 ml of 0.15 M acetic acid, 1.25 ml of chloroform and 1.25 ml of water. After vigorous mixing and centrifugation, the lower chloroform fractions (containing lipids) were collected, dried under a stream of N_2 and dissolved in 1 ml of chloroform.

To analyse the individual lipid classes and determine their fatty acids content and composition, aliquots of obtained chloroform fractions from individual seeds (3-4 seeds) at given DAP were mixed and separated by thin-layer chromatography on silica gel 60 plates (Merck), using hexane:diethyl ether:acetic acid (70:30:1; v:v:v) as the solvent system (“neutral solvent”). Separated lipids classes were visualized by spraying with water (even short exposure to I_2 vapours destroyed αESA; Fig. S1) and identified by means of standards. Marked gel fragments containing appropriate lipid classes were removed and transmethylated *in situ* on gel by adding 2 ml of 0.1 M NaOH in dry methanol (5 min at 90 ^{o}C). After incubation, internal standard (heptadecanoic acid methyl ester) was added together with 3 ml of hexane and 2 ml water. Following vigorous shaking and centrifugation the hexane fractions, containing fatty acid methyl esters, were collected and analysed by gas-liquid chromatography equipped with flame ionization detector (FID) and a WCOT fused-silica 50 m x 0.32 mm ID coating CP-Wax 58-CB DF 5 0.2 capillary column (Chrompack International, Middleburg, The Netherlands).

To analyse fatty acids content and composition of total acyl-lipids present in the chloroform extracts, aliquots of these extracts were dried under a stream of N_2, transmethylated and analysed on GC as described above.

Lipids of microsomal fraction were generally extracted and analysed as described above with some modifications presented in p.2.5.

Preparation of microsomal membrane

Seeds at 26 DAP were used for isolation of the microsomal fractions. Seeds' coats were manually removed and the embryos were placed in glass homogenizer and grinded with the addition of 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mg/ml of bovine serum albumin, 0.33 M sucrose and catalase (1000 U/ml). In the preliminary experiments (Fig. 4) homogenisation buffer contained additionally NADH (4 mM). The homogenates were filtered through two layers of Miracloth, diluted by fresh incubation buffer to 20 ml and centrifuged at 20,000 x g for 12 min. Obtained supernatants were collected and centrifuged again at 100,000 x g for 90 min. The resulting pellets (microsomal fractions) were washed with 0.1 M potassium phosphate buffer (pH 7.2) and homogenized with small volume of potassium buffer (in the preliminary experiments –Fig. 4 – microsomes were washed and resuspended in homogenization buffer). All stages of preparation of microsomal membranes were conducted at 4-5 ^{o}C and the isolated microsomal fractions were used directly for the assays (preliminary experiments; Fig. 4) or stored at -80 ^{o}C until further analysis. To determine the membrane concentrations in the obtained microsomal fractions aliquots of the suspensions were used for phosphatidylcholine (PC) content analyses.

Enzyme Assays

At the research described in p.3.4 reaction mixtures contained aliquots of freshly prepared microsomal fraction (140 nmol of microsomal PC, approximately 616 µg of microsomal proteins), 20 nmol of [^{14}C]acyl-CoA ([^{14}C]18:1-CoA, [^{14}C]18:2-CoA or [^{14}C]18:3-CoA), NADH (4 mM), BSA (1 mg/ml), 0.33M sucrose and catalase (1,000 U/ml) in 1 ml of 0.1 M p-buffer. In other experiments reaction mixtures contained aliquots of microsomal fractions (usually containing 24 nmol of endogenous PC - approximately 106 µg of microsomal proteins, unless stated differently), 10 nmol [^{14}C]18:2-CoA (if no stated differently), NADH (4 mM, in assays with NADH) and BSA (1 mg/ml) in 0.1 ml of p-buffer (pH 7.2). Reactions were carried out at 30 ^{o}C at different times (depending on the experiments) with shaking (1,250 rpm). Reactions were terminated by addition of 375 µl chloroform/methanol (1:2, v/v), 5 µl of glacial acetic acid, 125 µl of chloroform and 125 µl of water (in assays presented in p.3.4. 10 x bigger volume); modified Bligh and Dyer [21] method of lipid extraction.

Extracted microsomal lipids were directly separated on TLC with "neutral solvent" or with "polar solvent" - chloroform:methanol:acetic acid:water" (85:15:10:3.5; v:v:v:v) depending on the experiment. Chloroform extracts or separated lipid classes were transmethylated and analysed on GC as described in p.2.3 or

after transmethylation separated on TLC impregnated with $AgNO_3$ with hexane: diethyl ether: acetic acid (85:15:1; v:v:v) to separate 18:1-Me/αESA-Me, 18:2-Me and 18:3-Me from each other. The reaction products of added [^{14}C]-labelled substrates were visualized and quantified on TLC using electronic autoradiography (Instant Imager, Packard Instrument Co.).

All assays were done at least in duplicates and in the “results” average values or most representative chromatograms are presented.

Declarations

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Author contribution

The authors contributed equally to this work.

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Competing interests

The authors declare no competing interests.

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Tables

Table 1. Changes in fatty acid composition of complex lipids of microsomal fraction of developing seeds of *Momordica charantia* during incubation with and without NADH

Incubation time [min]	NADH [6 mM]	FA [mol%]				
		16:0	18:0	18:1	18:2	αESA
0	-	10.2	14.6	8.3	56.0	9.0
10	-	10.2	14.5	8.1	56.1	8.9
10	+	10.1	14.6	5.9	55.7	10.5
30	-	10.0	14.3	8.2	56.8	8.7
30	+	10.2	14.5	5.2	55.0	13.0

Mean values from duplicates are presented. In the assays microsomal fractions (aliquots contained 112 nmol microsomal PC; about 612 nmol of FA of all acyl-lipids) were incubated with and without addition of NADH. No other exogenous substrates was in the assays.

Figures

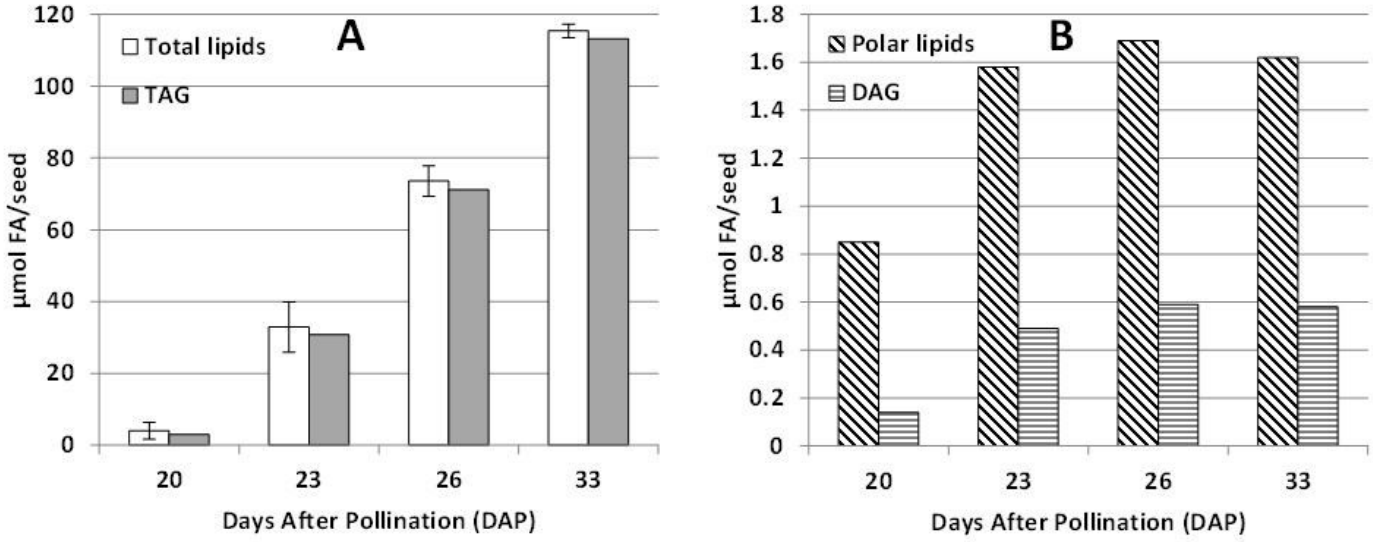

Figure 1

Accumulation of acyl lipids in developing Momordica charantia seeds. A – total lipids and triacylglycerol (TAG); B – polar lipids and diacylglycerols (DAG) Mean value and standard deviation (n≥3) are presented in case of total lipids; mean value for TAG, DAG and polar lipids (values obtained by multiplication of mean percentage amount of these lipid classes in total lipids by the amount of mean total lipids).

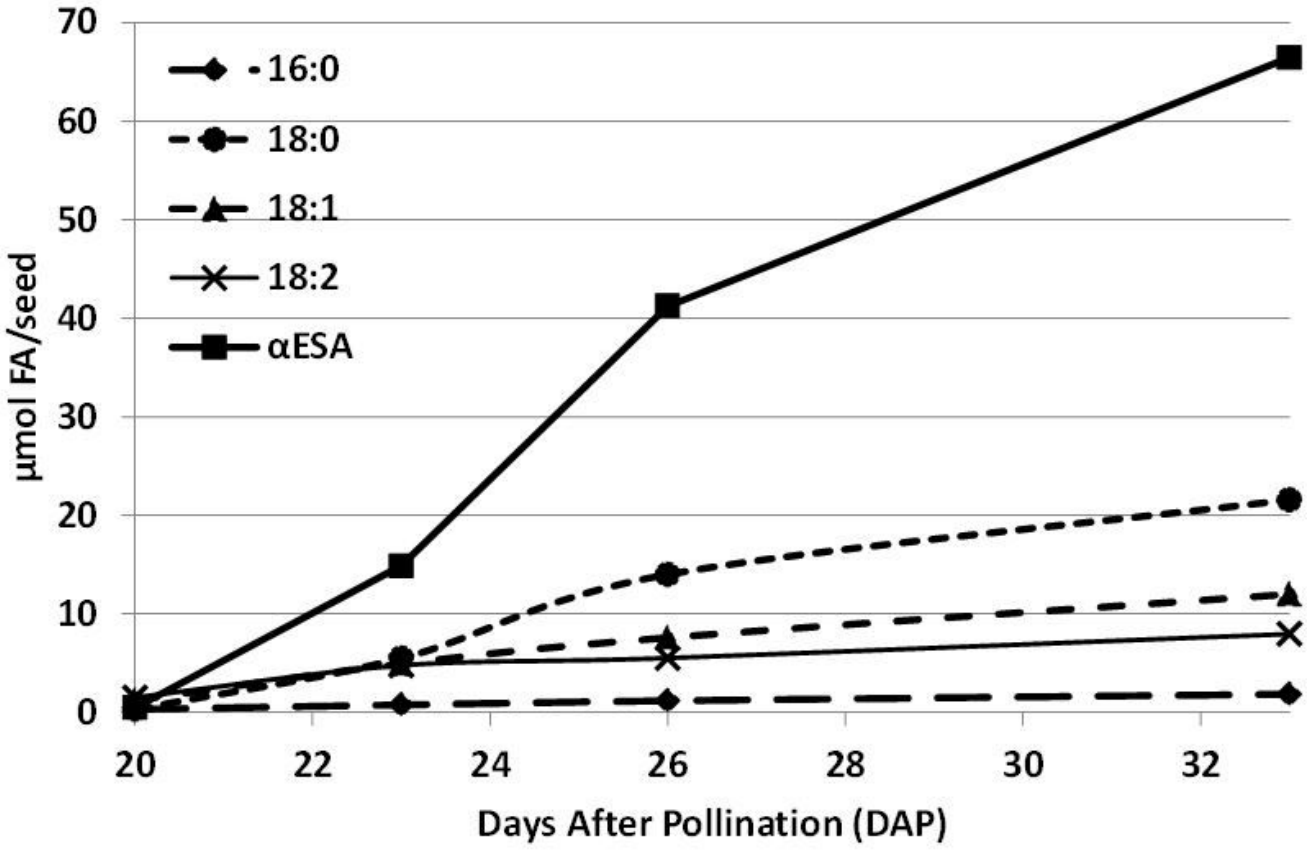

Figure 2

Accumulation of different fatty acids in acyl lipids of Momordica charantia developing seeds. Values obtained by multiplication of mean percentage amount of different fatty acids in total fatty acids by the mean amount of total fatty acids.

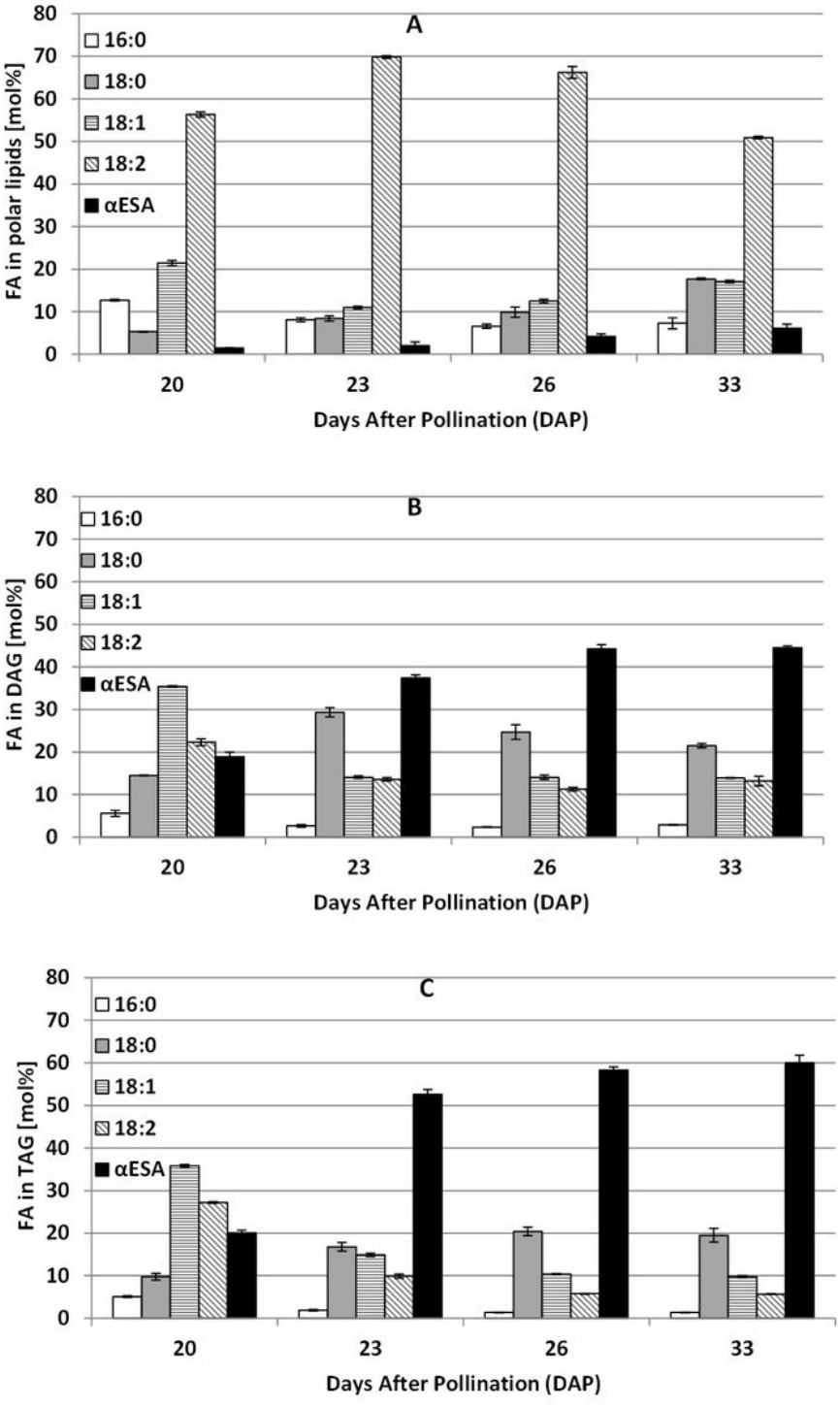

Figure 3

Fatty acid composition of polar lipids (a), diacylglycerols (b) and triacylglycerols (c) of Momordica charantia developing seeds.

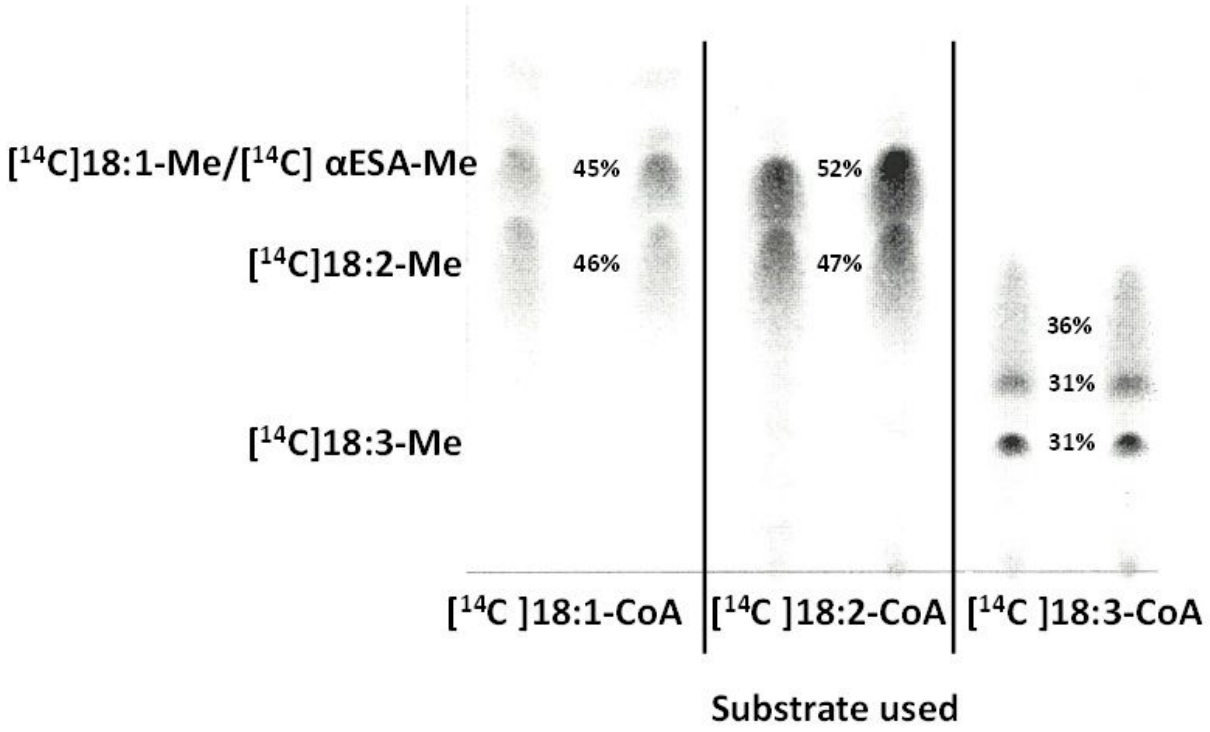

Figure 4

Products of added [14C]acyl-CoA formed during incubation with microsomal fraction of developing seeds of Momordica charantia. X% = average percentage of [14C] per line. Assays conditions: 20 nmol [14C]acyl-CoA, BSA (4 mg/ml), NADH (4 mM); incubation time: 120 min.; aliquots of microsomal fractions: 142 nmol microsomal PC. Used [14C]18:2-CoA contained about 20% of impurities localised on the plate together with [14C]18:1-Me/[14C] αESA-Me.

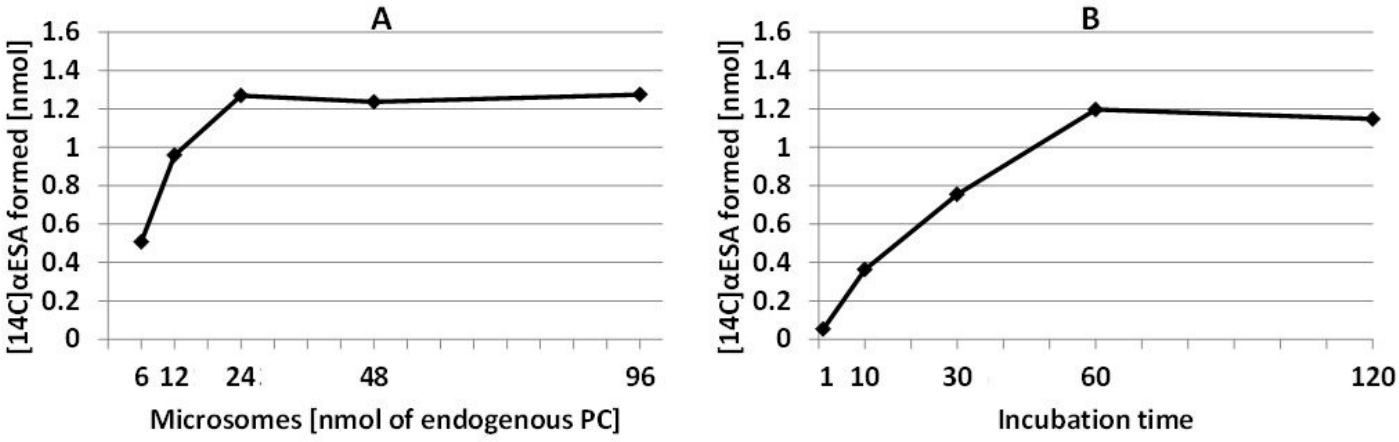

Figure 5

The impact of different amounts of microsomes [A] and incubation time [B] on the effectiveness of αESA biosynthesis by microsomal fraction of developing seeds of Momordica charantia. Assays conditions: 10nmol [14C]18:2-CoA, BSA (4 mg/ml), NADH (4 mM); incubation time: a – 60 min., b – as indicated on the figure; aliquots of microsomal fractions: a – as indicated on the figure, b – 24 nmol endogenous PC.

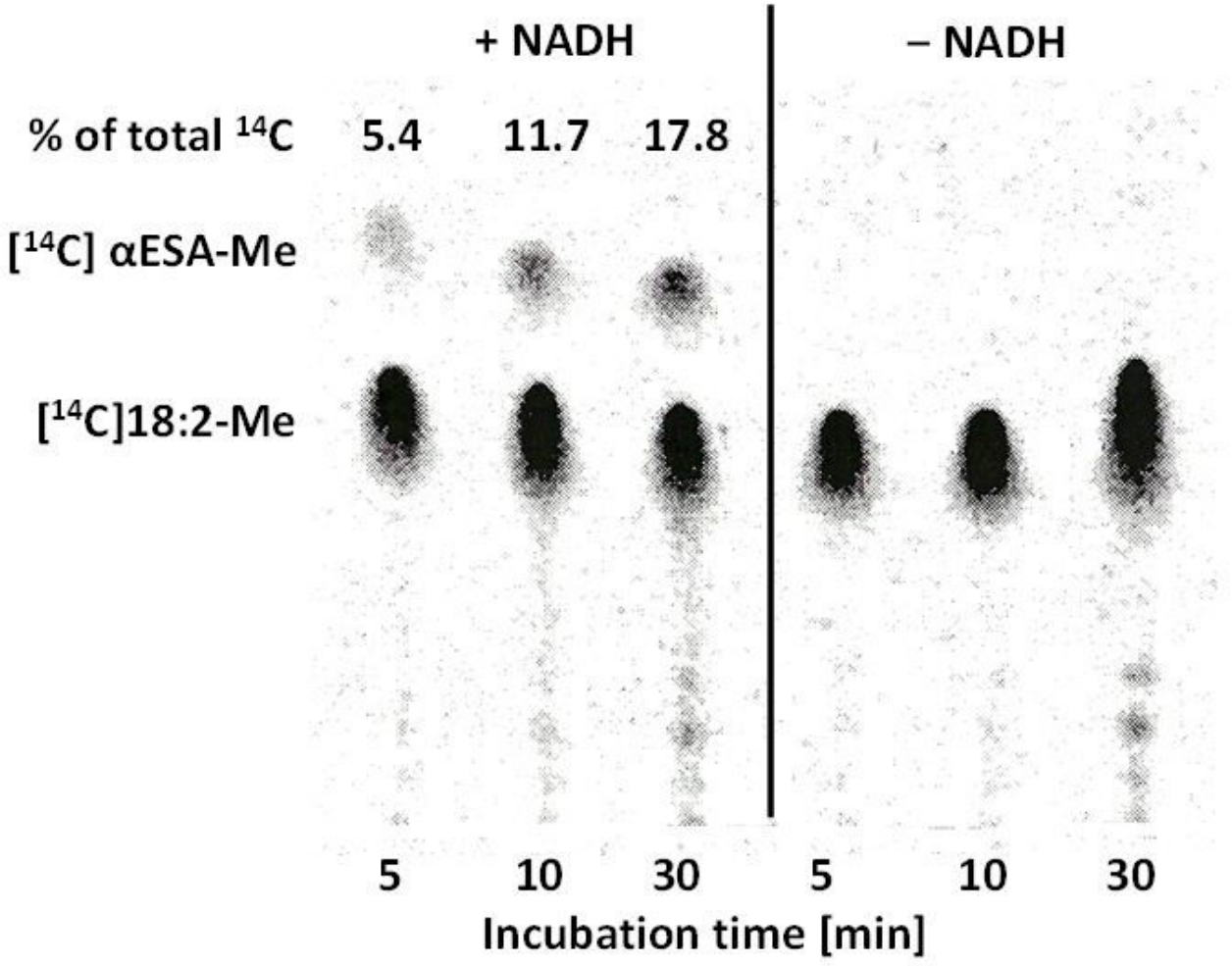

Figure 6

Biosynthesis of [14C]αESA by microsomal fraction of developing seeds of Momordica charantia from exogenous [14C]18:2-CoA in the presence and absence of NADH. Assays conditions: 10 nmol [14C]18:2-CoA, BSA (4 mg/ml), NADH (4 mM in assays with NADH); aliquots of microsomal fractions: 24 nmol of microsomal PC.

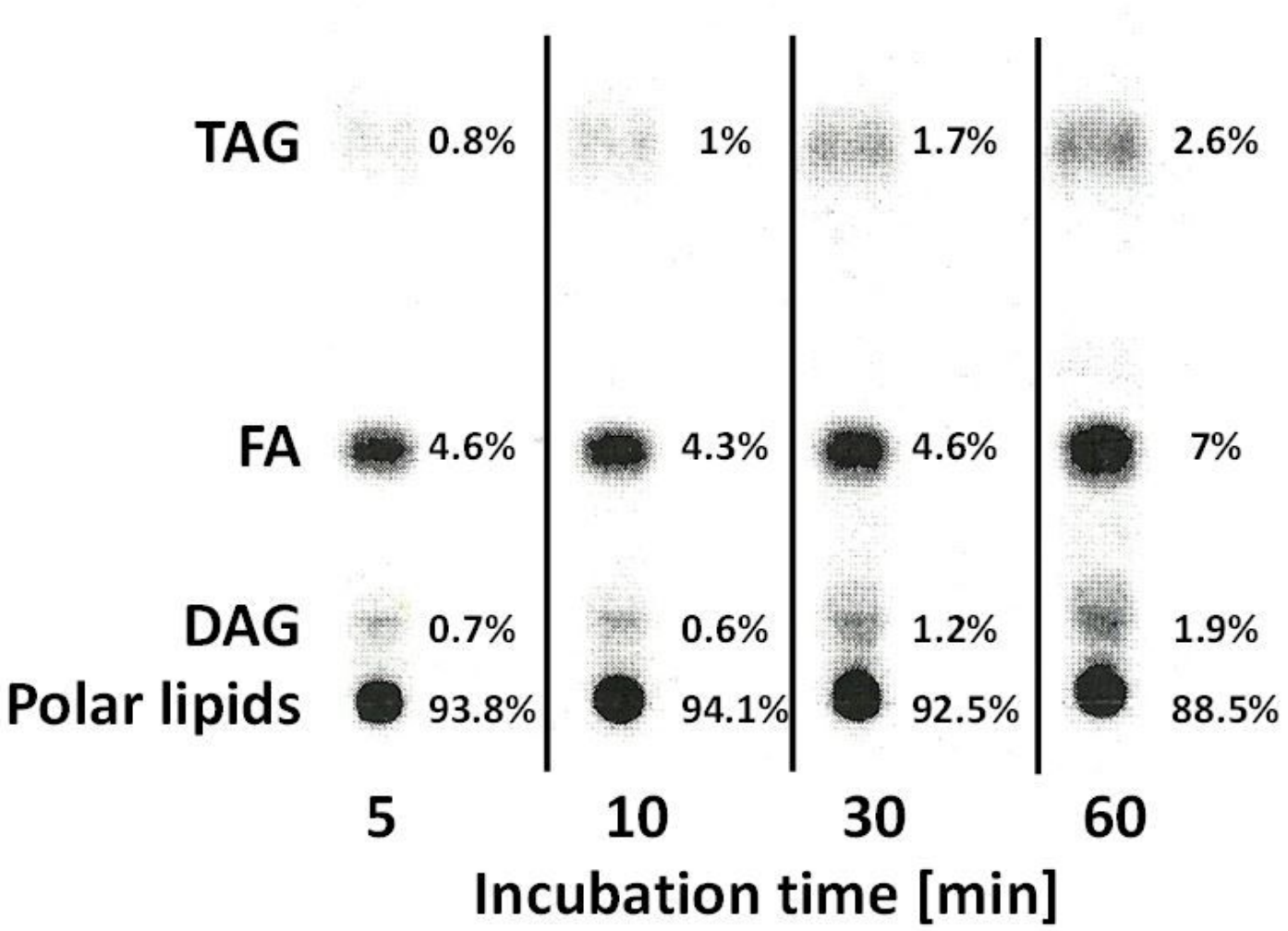

Figure 7

Distribution of radioactivity from added [14C]18:2-CoA by different lipid classes (separated on TLC with neutral solvent) during incubation of microsomal fraction of developing seeds of Momordica charantia. X% = percentage of [14C] per line. Assays conditions: 10 nmol [14C]acyl-CoA, BSA (4 mg/ml), NADH (4 mM); aliquots of microsomal fractions: 24 nmol of microsomal PC.

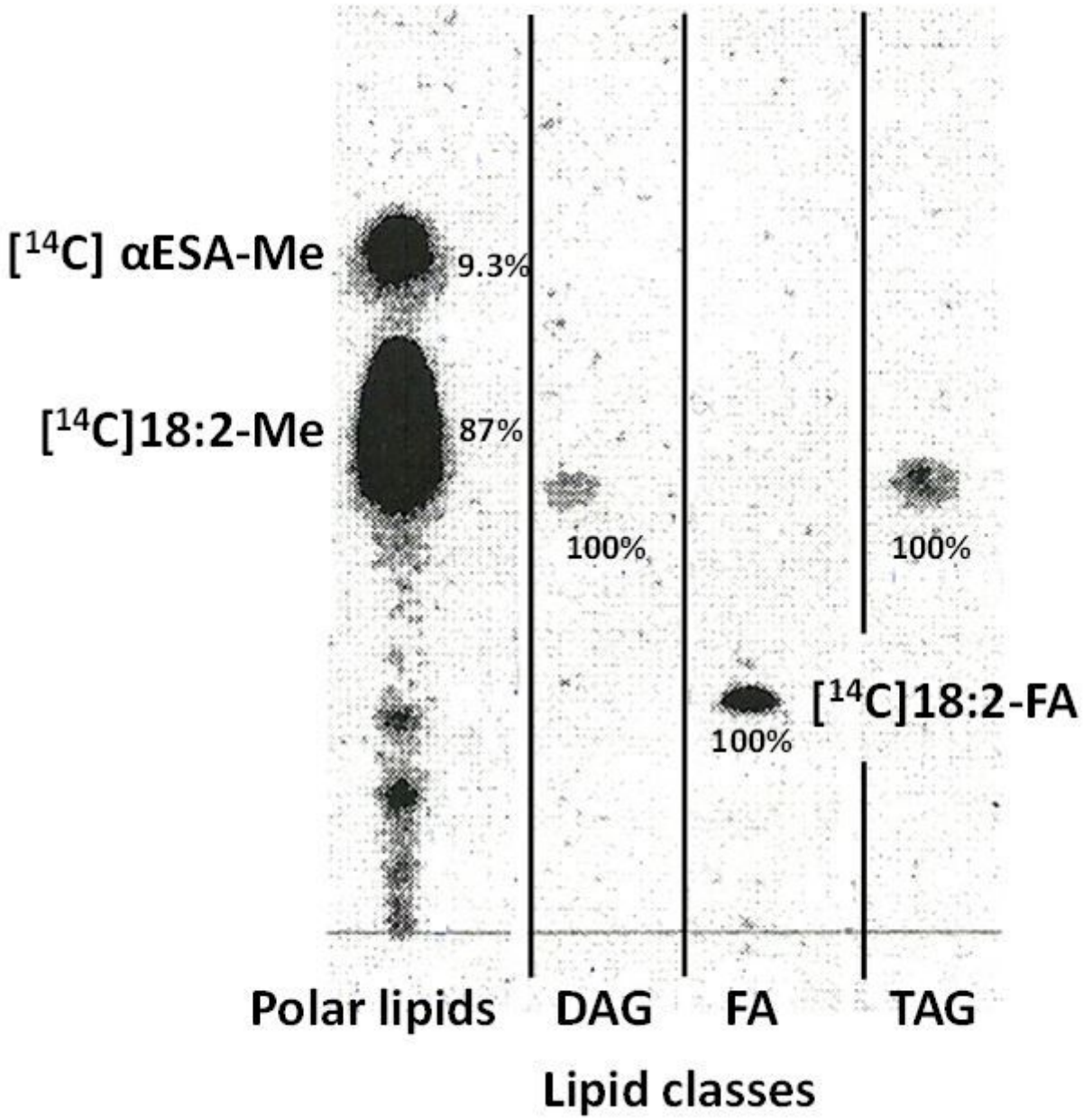

Figure 8

Distribution of [14C]αESA and [14C]18:2 by different lipid classes after 10 min incubation of microsomal fraction of developing seeds of Momordica charantia with exogenous [14C]18:2-CoA. X% = percentage of [14C] per line. Assays conditions: 10 nmol [14C]acyl-CoA, BSA (4 mg/ml), NADH (4 mM); aliquots of microsomal fractions: 24 nmol microsomal PC.

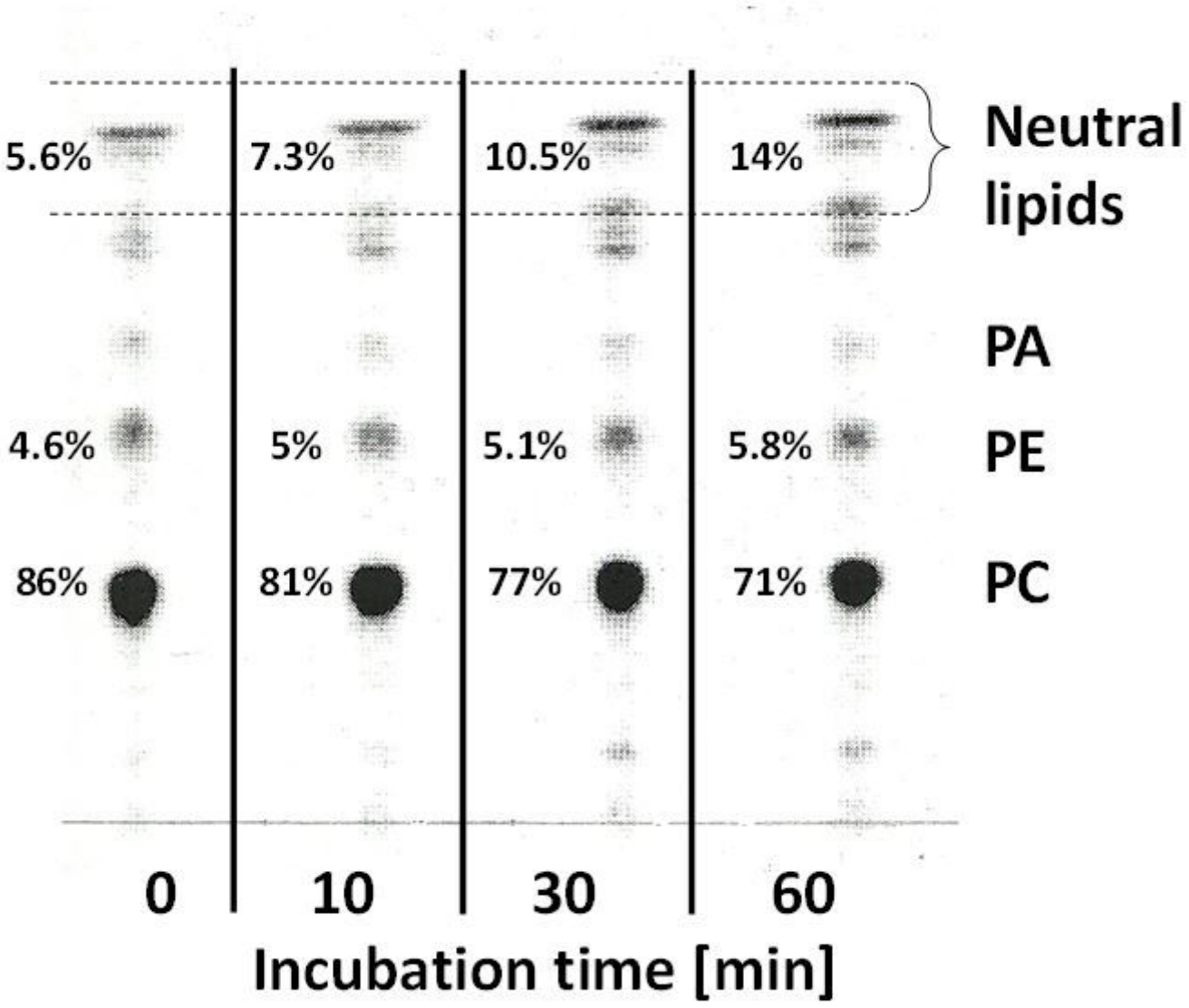

Figure 9

Distribution of radioactivity from added [14C]18:2-CoA by different lipid classes (separated on TLC with polar solvent) during incubation of microsomal fraction of developing seeds of Momordica charantia. Microsomes were pre-incubated for 10min with [14C]18:2-CoA without NADH (indicated on graph as 0 min incubation time). Afterwards a new buffer with NADH was added to the pelleted microsomes and incubated for the remaining time. X% = percentage of [14C] per line.

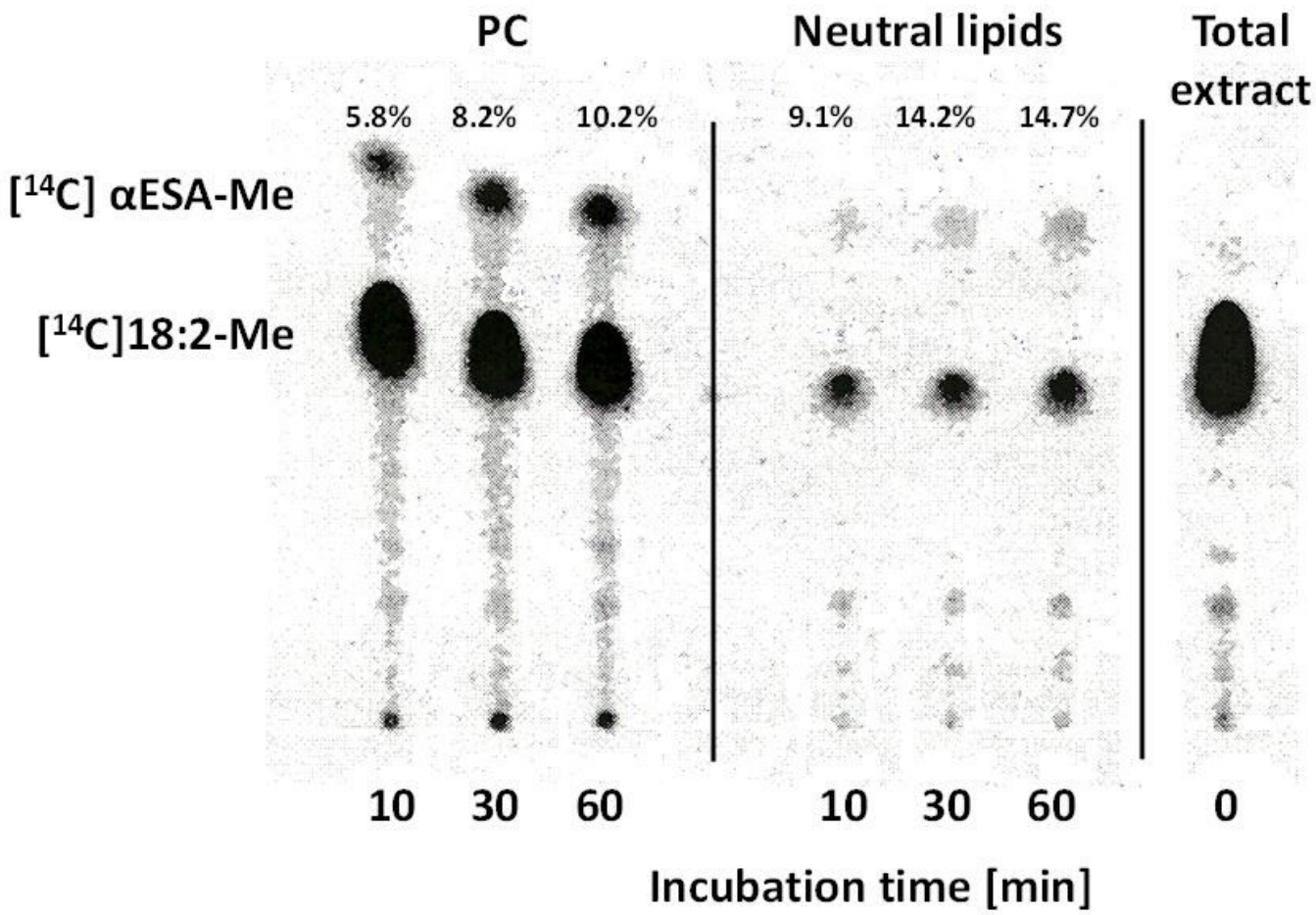

Figure 10

Distribution of [14C]αESA and [14C]18:2 in PC and neutral lipids during incubation of microsomal fraction of developing seeds of Momordica charantia. Microsomes were pre-incubated for 10min with [14C]18:2-CoA without NADH (indicated on graph as 0 min incubation time). Afterwards a new buffer with NADH was added to the pelleted microsomes and incubated for the remaining time. X% = percentage of [14C] per line.

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