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Purification and analysis of a low density membrane fraction from developing seeds of *Brassica napus*

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Abstract

A low density membrane fraction (LDM) identified in developing embryos of *Brassica napus* was previously shown to contain relatively high activities of the enzymes of triacylglycerol (TAG) synthesis compared to those involved in phospholipid synthesis. The LDM fraction used in that study was highly contaminated with non-membrane proteins. In this study, the LDMs were purified by linear sucrose gradients followed by flotation through a discontinuous sucrose gradient that removed most detectable contaminating proteins. The data presented in this work show that the low density membrane vesicles were purified over 250-fold with respect to the specific activities of the enzymes of TAG synthesis. The specific activity of TAG biosynthesis in LDMs was over 280 times higher than TAG synthesis by purified endoplasmic reticulum. Analysis of the polypeptide composition by SDS-PAGE of the crude and purified LDMs revealed that the purification process had removed a large number of contaminating proteins. The predominant polypeptides of the LDMs were also found in the ER fraction but a number of the main ER proteins were missing from the purified LDMs.

Keywords: *Brassica napus*; Endoplasmic reticulum; Triacylglycerol; Acyltransferase; Seed (developing)

1. Introduction

Vegetable oils are an important agricultural commodity for the food and non-food industries. Although the metabolic pathways involved in oil synthesis are well known, the mechanism of deposition of oil as oil bodies is not so well understood (Napier, Stobart, & Shewry, 1996). It is generally thought that storage oil is synthesised by the ER and in some way secreted from the membrane coated with oleosins which prevent coalescence of the oil during the desiccation/rehydration of the seed (Abell et al., 1997; Leprince, van Aelst, Pritchard, & Murphy, 1998). It was previously shown that triacylglycerols were synthesised by two discrete membrane fractions in homogenates made from developing embryos of oilseed rape (Lacey & Hills, 1996). Similar heterogeneity of the endoplasmic reticulum with respect to the distribution of acyltransferases has also been observed in lactating mammary gland (Valivullah, Dylewski, & Keenan, 1986). The membranes were isolated on sucrose density gradients and although much of the capacity for the synthesis of TAG was shown to co-localise with the ER marker enzymes such as DAG: cholinephosphotransferase and lysophosphatidylcholine acyltransferase, a low density membrane fraction which synthesised TAG was shown to have substantially lower activities of the ER markers. The LDMs contained much larger amounts of TAG and DAG than did the bulk ER. In the light of the relatively high activities of the TAG synthesis enzymes it was suggested that the LDMs might be derived from those parts of the ER which are most actively involved in the synthesis/secretion of TAG. Since relatively little is understood about the mechanism of TAG synthesis and oil body deposition, further study of these TAG-rich LDMs should shed light on the process. It was found that the low-density membranes isolated by the method previously described were heavily contaminated with soluble proteins. A number of strategies to purify the LDMs had previously been tried and the polypeptide composition of the LDMs analysed by SDS-PAGE (Lacey, Pongdontri, & Hills, 1997). N-Terminal sequencing of the most prominent polypeptide bands revealed that the membranes were still heavily contaminated with soluble proteins such as cruciferin and Rubisco amongst others (Lacey et al., 1997). This paper describes a strategy that substantially purifies the LDMs in terms of enzyme activity and protein composition and

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provides evidence that the LDMs are likely to have been derived from the ER.

2. Results and discussion

2.1. Purification of the low density membranes by flotation

Low-density membranes and ER were isolated by centrifugation on a linear sucrose density gradient as previously described (Lacey & Hills, 1996). For further purification of the LDMs, the fraction was brought to a sucrose density of 30% w/w, overlaid with 20% and then 5% w/w sucrose solutions and centrifuged as described in Section 3. The LDMs floated up through the 30% sucrose and rested at the interface between the 5 and 20% sucrose layers. The protein content, activities of the acyltransferases incorporating oleoyl groups into TAG and the other glycerolipid intermediates of the Kennedy pathway (diacylglycerol, phosphatidic acid and lysophosphatidic acid) and acyl-CoA thioesterase were determined in the 30, 20 and 5% sucrose fractions and membranes from the 20/30% and 5/20% sucrose interfaces. For clarity only the results from the purified LDMs, the soluble proteins and the original LDM preparation are given (Table 1). Only the LDM fraction at the 5/20% sucrose interface incorporated [1-^{14}C]oleoyl moieties into glycerolipids and no incorporation into lipids was detected in any other fraction. The soluble acyl-CoA thioesterase activity was detected only in the 30% sucrose fraction as expected. This was an indication that the LDMs had been isolated very well from the contaminating proteins of the cytosol. The purified LDMs incorporated over 60% of the total [1-^{14}C]oleoyl moieties into TAG compared to the unpurified LDM fraction where 40% was incorporated. The protein content of the purified LDM vesicles was very low compared to that remaining in the 30% sucrose fraction Table 1. The TAG synthesis activity in the LDMs was purified over 250-fold from the crude LDM fraction on a protein basis. The specific activity of incorporation of [1-^{14}C]oleoyl moieties from [1-^{14}C]oleoyl-CoA by the purified LDMs was 53.9 nmol mg^{-1} protein min^{-1}. For comparison, this is about 50–100 fold higher than the specific activity determined for the peak ER fraction of sucrose density gradients made from rape embryo homogenates which ranged from 0.3 to 0.7 nmol mg^{-1} protein min^{-1} in five experiments (Lacey, 1996; Lacey & Hills, 1996). The activities of the acyltransferases and thioesterase at the 20/30% sucrose interface were identical to those measured for the 30% fraction, as would be expected. Protein was not detectable in the 20 and 5% sucrose layers.

2.2. Polypeptide composition of purified low-density membrane vesicles

Since the TAG synthesis activity of the purified LDMs was so much higher than that of the endoplasmic reticulum, it would be expected that the polypeptide composition will also be substantially different. Proteins from the fractions obtained during LDM purification and peak ER fraction from the linear sucrose gradient were resolved on 8–15% SDS-PAGE and visualised by silver staining since the amount of purified LDM protein produced was very small (Figure 1). The polypeptide composition of purified LDMs was very different to that pre-purification (lanes 5 and 2, respectively). There were 12 prominent polypeptides in the LDM fraction ranging in M_r from 21–82 kDa. Polypeptides with identical molecular mass were also present in the peak ER fraction but the relative staining intensities of individual bands varied between the two fractions (lanes 5 and 6). These data corroborate the hypothesis that the LDMs are derived from the ER, though having their own particular pattern in amounts of any particular polypeptide. In addition, there were many more polypeptides in the ER fraction, which were not present in the purified LDMs. It is possible that some of the polypeptides observed in the ER fraction were derived from other membrane systems such as Golgi, plasma and vacuolar membranes that co-localise with the ER. Generally, however, these membranes are much less abundant than the ER. In future work it will be useful to target some of the prominent polypeptides in the LDM fraction for further study.

Table 1
TAG and glycerolipid synthesis, acyl-CoA thioesterase and protein content in low-density membranes purified from developing seeds of *B. napus*

Fraction	Incorporation of oleate into lipid (nmol mg^{-1} protein min^{-1})		Protein (μg)	
	TAG	FA	total glycerolipid[a]	
Total LDM	0.078	0.175	0.197	3200
Purified LDM	33.0	n.d.	53.9	2
Soluble	n.d.	0.168	n.d.	3200

LDM, low-density membrane; n.d., not detectable.

[a] Acyl groups incorporated into triacylglycerol, diacylglycerol, phosphatidic acid and lysophosphatidic acid. Acyl-CoA thioesterase determined by amount of free fatty acid detected.

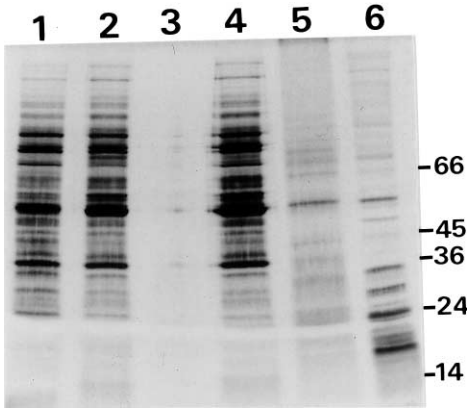

Fig. 1. 5 μg of protein (or the entire purified LDM fraction, approximately 2 μg) were resolved by 8–15% SDS-PAGE and visualised by silver staining. Lane 1: soluble protein; lane 2: unpurified LDM; lane 3: 20/30% interface; lane 4: 30% sucrose cushion (soluble protein); lane 5: purified LDMs and lane 6: peak ER fraction from linear gradient. The molecular mass markers are shown on the right side in kDa.

3. Experimental

3.1. Plant Material

Developing seeds of *Brassica napus* L. cv Topas were harvested from glasshouse grown plants at the stage of maximum oil synthesis as previously described (Lacey & Hills, 1996).

3.2. Purification of low-density membranes

The developing seeds were homogenised and low-density membrane and endoplasmic reticulum fractions isolated by sucrose density gradient centrifugation as previously described (Lacey & Hills, 1996). The LDM fraction was further purified by modifications to a flotation procedure previously described (Coughlan, Hastings, & Winfrey, 1996). The peak LDM fraction from the sucrose gradient was mixed with vol. of a buffer containing 48% (w/w) sucrose, 5 mM EDTA, 1 mM PMSF, 10 mM KCl, 2 mM DTT and 100 mM Hepes (NaOH, pH 7.5), to give a final sucrose concentration of 30% (w/w). The sample was overlaid with 2 ml of a similar buffer containing 20% (w/w) sucrose and finally 1 ml of the buffer containing 5% (w/w) sucrose. The LDMs were floated by centrifugation at $100{,}000 \times g$ for 16 h in a swing-out rotor. The LDMs were collected at the 5–20% interface with a Hamilton syringe in a volume of 300 μl. Other regions of the step gradient were also removed for analysis.

3.3. Enzyme and protein analysis

Incorporation of [1-^{14}C]oleoyl moieties from [1-^{14}C]oleoyl-CoA into triacylglycerol and the intermediates of the Kennedy pathway were determined as previously described (Lacey & Hills, 1996). Protein was estimated by the method of Bradford (1976). Proteins were separated by SDS-PAGE using a Mini-Protean dual slab cell (Bio-Rad, Watford, UK) on 8–15% gels according to the manufacturers instructions. The polypeptides were visualised by silver staining (Oakley, Kirsch, & Morris, 1980).

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