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SEED OIL-BODIES: ISOLATION, COMPOSITION AND ROLE OF OIL-BODY APOLIPOPROTEINS

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Key Word Index—Apolipoproteins; oil-bodies; oilseeds; oleins; triacylglycerols.

Abstract—A simple, rapid procedure for the isolation of purified seed oil-bodies is reported. The oil-bodies were collected by two floatation steps following centrifugation of tissue homogenates. The oil-bodies from a variety of oilseed species contained *ca* 80% triacylglycerol, 15% apolipoprotein and 2% phospholipid. Further purification steps did not alter these proportions but did reduce considerably the final yield of oil-bodies. Electron microscopic and gel electrophoretic examinations also showed that only two floatation steps were required to produce a pure oil-body fraction. The oil-body apolipoprotein was tightly bound to the oil-body surface and could not be removed by washing. The oil-bodies and their associated apolipoproteins were also resistant to prolonged incubation with a variety of ionic and non-ionic detergents, including Triton X-100, deoxycholate, CHAPS and SDS. Delipidation of the oil-bodies with organic solvents resulted in the formation of oil-body 'ghosts' if diethylether were used, or membranous 'tubules', if chloroform were used. Purified triacylglycerols were readily reconstituted *in vitro* into oil-bodies following sonication in the presence of purified oil-body membranes or components thereof. The role of the apolipoproteins in seed oil-bodies is discussed.

INTRODUCTION

Oilseed crops have tremendous potential as sources of both edible oils and renewable oil-based raw materials for the chemical and pharmaceutical industries. Many oilseed crops are also good sources of high quality protein for both livestock and human consumption. Despite the ever-expanding worldwide use of oilseed products [1], much remains to be learned about the basic processes by which storage oils are formed. Seed storage oils are synthesized during seed development [2, 3] either in the embryonic tissue, as in rapeseed, sunflower seed or soybeans, or in another tissue as in castor bean (endosperm). In all cases, the seed oil is eventually deposited into oil-bodies, which generally range from 0.4 to 1.5 μm average diameter in the mature seed tissue. The nature and formation of oil-bodies has been debated by numerous authors but hitherto no consensus view has emerged [2–10]. In particular, the question of the presence and amount of protein associated with oil-bodies has been a controversial one [2, 3, 6, 10–13]. Estimates of the amount of oil-body protein have varied from 0.2 to 32% w/w. These discrepancies may be largely due to differences in procedures for oil-body isolation and protein estimation. While investigating the mechanism of oil-body formation in several common oilseed species, we noticed that relatively large amounts of protein were invariably associated with purified oil-bodies, providing that a standard isolation procedure was employed and that oil-bodies were delipidated in neutral solvents prior to protein estimation. These hydrophobic oil-body proteins may share many properties with the apolipoproteins which form a boundary membrane around mammalian chylomicrons. Like seed oil-bodies, the latter are also triacylglycerol-containing bodies of 1–2 μm diameter [14, 15]. In this communication, we report the development of a simple rapid procedure for the isolation of pure, intact seed oil-bodies. The characteristics of the integral oil-body membrane apolipoproteins are examined and the reconstitution *in vitro* of oil-bodies is reported.

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RESULTS

Isolation and composition of oil-bodies

Oil-bodies are conventionally isolated by repeated floatation following centrifugation of tissue homogenates [4–13]. Rapeseed oil-bodies, which had been subjected to four cycles of floatation/resuspension, were found to consist of about 80% triacylglycerols, 15% apolipoproteins and 2% phospholipids, as shown in Table 1. Similar results were found with other oilseed species such as *Brassica oleracea* (cabbage), *Sinapis alba* (mustard), *Raphanus sativa* (radish), *Arabidopsis thaliana*, *Glycine max* (soybean), *Helianthus annuus* (sunflower) and *Zea mays* (maize). The oil-body fractions had a relatively high protein content which compared favourably with the values reported for other oilseed species, such as soybean (15–30% protein [6]), crambe (19% [16]) and safflower (11% [16]) although it is higher than the values reported for linseed (2.3% protein [13]), sunflower (4.3% [16]) and peanut (1.3% [11]). It is possible that some of these variations are due to the poor recovery of protein from non-delipidated oil-bodies and to the use of different types of protein assay.

The integral nature of the repeseed oil-body apolipoproteins is demonstrated in Fig. 1 where the recovery of

Table 1. Lipid and protein compositions of mature cotyledons and purified oil-bodies from rapeseed

	Lipid (mg/embryo)			Protein (mg/embryo)			
	Total	PL	TG	Total	Storage*	Apolipoprotein†	Other
Mature cotyledons	2.05	0.10	1.95	1.17	0.75	0.35	0.07
Oil-bodies‡	1.66	0.04	1.62	0.29	0	0.29	0

*Storage proteins are the 'soluble' proteins, cruciferin and napin.
†Apolipoproteins refers to the intrinsic protein fraction associated with the oil-body fraction.
‡The oil-body fraction analysed was OB2 (see Experimental).
Lipids were fractionated by TLC and estimated by GC of methylester derivaties. Proteins were estimated by Lowry and Bradford assays and by PAGE/densitometry.

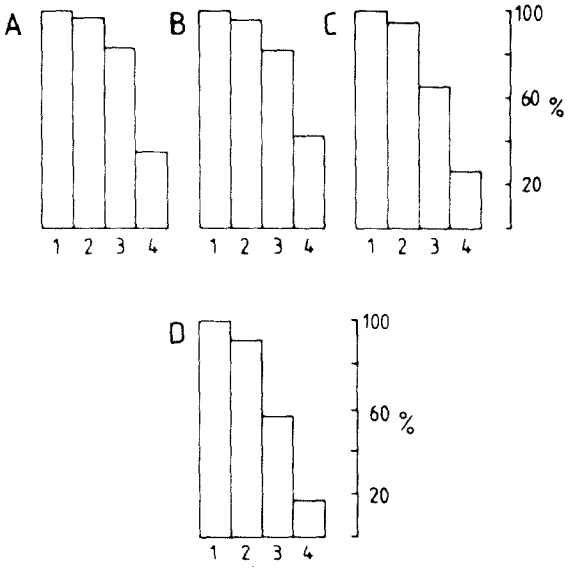

Fig. 1. Recovery of protein and oil at different stages during isolation of intact oil-bodies from mature rapeseed. (1) 1st oil-body fraction (OB1); (2) 2nd oil-body fraction (OB2); (3) 3rd oil-body fraction (OB3); (4) 4th oil-body fraction (OB4). Protein was estimated by (A) SDS-PAGE/densitometry; (B) modified Lowry assay [18]; (C) Bradford assay [17]. Total lipid (D) was estimated gravimetrically or by GC.

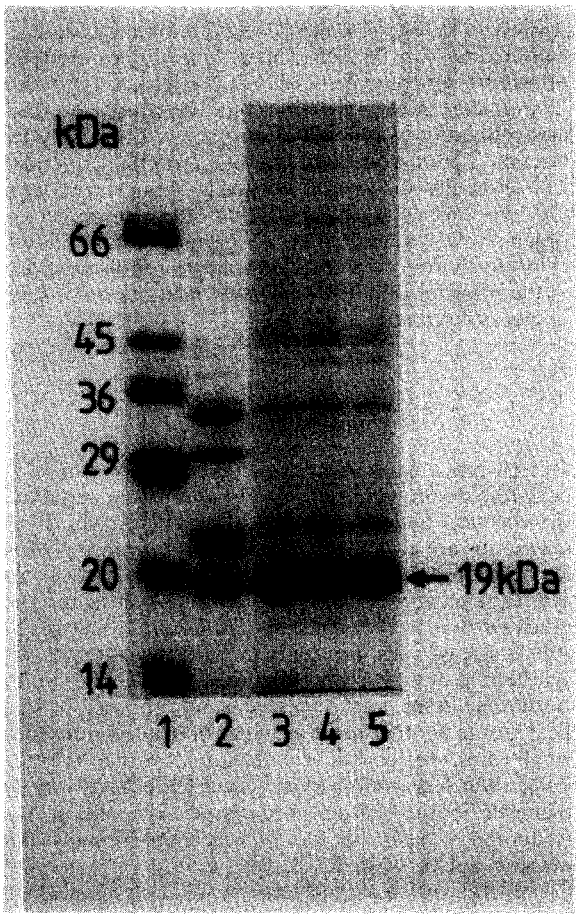

Fig. 2. Polypeptide composition of fractions from different stages during isolation of intact oil-bodies from mature rapeseed. Tissue extracts were delipidated (× 3) in diethylether and polypeptides separated on 15% polyacrylamide gels. Lane 1, M_r markers; lane 2, total seed homogenate; lane 3, 1st oil-body fraction; lane 4, 2nd oil-body fraction; lane 5, 3rd oil-body fraction.

protein and oil is compared after the various floatation stages. The results from three independent protein assays showed that the decline in the amount of apolipoprotein at each stage of purification was very closely matched by the decline in the amount of oil. This shows that there was no selective loss of protein and that the decline in yield was due to the loss of whole oil-bodies. These data are confirmed by electrophoretic analysis of the various oil-body fractions as shown in Fig. 2. This shows that even the crude oil-body fraction (OB1) was highly enriched in the characteristic 19 000 oil-body apolipoprotein [10, 17, 18] and was relatively uncontaminated by soluble seed storage proteins. The second oil-body fraction (OB2) was slightly purer than OB1, as judged by its polypeptide composition, while further purification steps (OB3 and OB4) made little difference to the purity but substantially reduced the total yield of oil-bodies. We conclude that a maximum of two floatation steps is sufficient to obtain a pure oil-body fraction from rapeseed. This was also found to be true for other oilseed species, such as cabbage, mustard, radish and Arabidopsis (results not shown).

Ultrastructure of oil-bodies

The above conclusion was reinforced by the results of electron microscopic (EM) studies. Mature rapeseed oil-

bodies *in situ* have diameters of between 0.4 and 1.5 μm but their average diameter is under 1 μm [10, 17, 18]. Isolated oil-bodies were found to have similar dimensions, as shown in Fig. 3. Mature rapeseed cotyledons are mostly made up of oil-bodies and soluble proteins—these two constituents together make up in excess of 84% of total cotylendon weight, with the balance largely made up of other soluble materials and fibres. It was therefore not surprising to find that even total homogenates of rapeseed cotyledons, when examined by EM, appeared to consist mostly of intact oil-bodies, with minimal contamination by other membranes. Mature oilseeds, such as rapeseed contain few, if any, endomembranes. The appearance under EM of the OB1–OB4 fractions varied very little and was similar to that shown in Fig. 3A.

Effect of detergents

The association between the rapeseed apolipoproteins and the oil-bodies was strong enough to withstand repeated washing in aqueous buffers. Most membrane proteins are, however, readily solubilized in the presence of excess detergent. The effects on oil-body structure of the detergents Triton X-100, sodium dodecyl sulphate (SDS), sodium deoxycholate (DOC) and 3-[(3-cholamido propyl)dimethylammonio]-1-propanesulphonate) (CHAPS) were examined. The detergents were incubated for 30 min at concentrations above their CMCs and at a detergent:protein ratio of 5:1 in large excess with intact oil-body preparations. In some cases, further detergent was then added and the mixtures incubated for another 30 min. In all cases, there was no discernable effect on

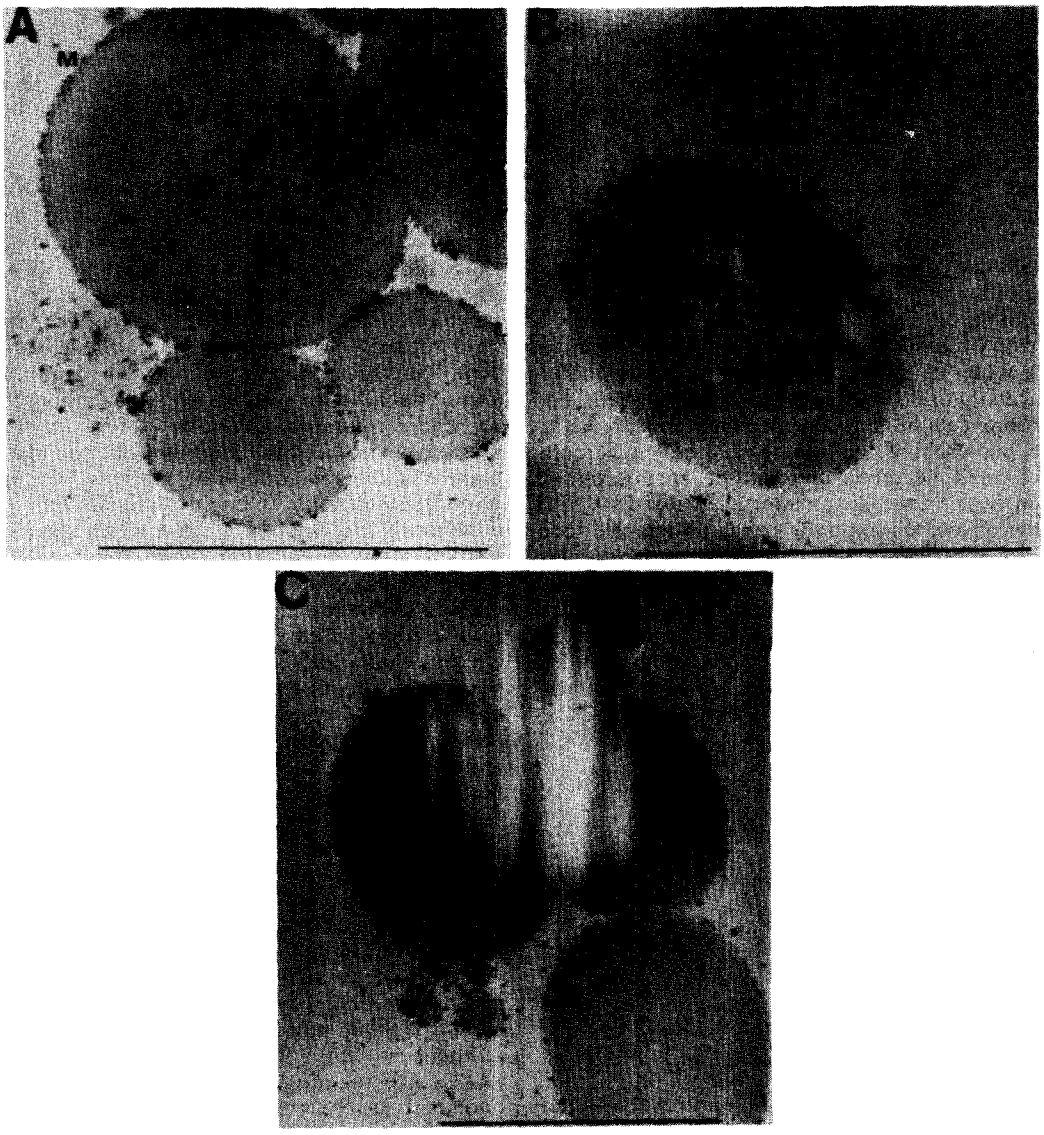

Fig. 3. Electron micrographs of intact oil-bodies isolated from mature rapeseed. (A). Oil-body fraction after three floatation steps (OB3). (B). OB3 after 30 min incubation at 25° with 0.61% Triton X-100. (C). OB3 after 30 min incubation at 25° with 0.25% SDS. Oil-bodies were suspended in 2% LMPagarose and processed for microscopy. O, storage oil; M, oil-body membrane. Bars represent 1 μm.

either oil-body ultrastructure (Fig. 3A–C) or on the composition of the oil-bodies. These results show that, unlike most biological membranes, the oil-body membrane was highly resistant to detergent attack, even when strongly ionic agents, such as SDS, were employed. It was possible to disrupt the oil-bodies with SDS only when elevated temperatures were used and, even then, several minutes in boiling SDS did not completely extract the oil-body apolipoproteins.

Delipidation of oil-bodies

The only agents capable of disrupting oil-bodies at room temperature were organic solvents and proteases. The effects of organic solvents and trypsin on the integrity of soybean oil-bodies [6] and of some organic solvents on mustard oil-bodies [19] have been reported. In the present study, it was found that three cycles of delipidation by organic solvents were required, as shown in Fig. 4. The best solvent was diethylether since this effectively removed virtually all of the neutral lipids but left behind the vast majority of the phospholipids and apolipoproteins. Chloroform–methanol (2:1) was also effective at removing neutral lipids but also removed much of the phospholipids plus a large proportion of the apolipoproteins. Chloroform alone was less effective at extracting either neutral lipids (10 times less efficient than diethylether) or phospholipids and apolipoproteins.

The effects of organic solvents on oil-body ultrastructure are shown in Fig. 5. Chloroform treatment did not remove all the oil, even after three washes, and in Fig. 5A two partially solubilized oil-bodies can still be discerned. The remainder of the section consists of tubular structures made up of phospholipid and apolipoprotein. The tubular remnants of the oil-body membranes are disorganised but each of the sectioned 'tubes' apparently contains a unit membrane on each side. Diethylether or chloroform–methanol (2:1) treatment removed virtually all of the oil but the oil-body remnants retained their structure apart from a slightly 'shrivelled' appearance (Fig. 5B). The oil-body ghosts, now devoid of oil were bounded by what looked like a double unit membrane. It is possible that this apparent 'double unit membrane' was not made up of two conventional bilayer membranes, but rather of two closely appressed apolipoprotein membranes containing minor amounts of phospholipid. Certainly the composition of the oil-body ghosts was over 90% protein with phospholipid making up most of the balance.

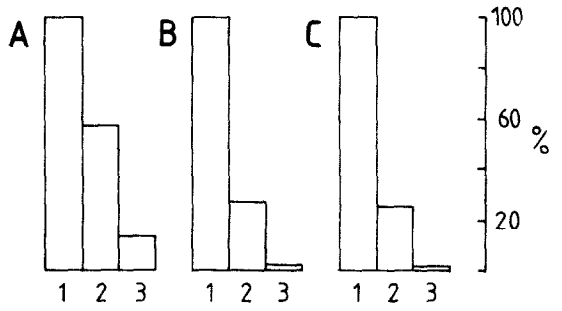

Fig. 4. Effect of delipidation in various organic solvents on the triaclyglycerol content of isolated rapesed oil-bodies. Oil-body fractions, isolated after two floatation stages (OB2) were subject to (1) one; (2) two; (3) three cycles of delipidation in 5 vols of (A) chloroform; (B) chloroform–methanol (2:1); (C) ether. Total triacylglycerols were separated by TLC and estimated by GC.

Oil-body apolipoproteins. The polypeptide composition of the apolipoprotein fraction of various species is shown in Fig. 6. As we have shown above, intact oil-bodies were resistant to detergent solubilization. It was necessary, therefore, to delipidate the oil-bodies prior to incubation with SDS, in order to obtain good resolution of polypeptides on PAGE gels. Note the predominance of low-M_r polypeptides of 17–22 000 amongst the apolipoprotein extracts of all the *Cruciferae* species. In the case of rapeseed, the 19 000 polypeptide makes up 60–70% total oil-body protein. We have recently found that these highly abundant 17–22 000 oil-body polypeptides form a single family of immunologically cross-reactive apolipoproteins which have been termed 'oleosins'. This result has been further confirmed by peptide mapping studies (Au, D. and Murphy, D. J. unpublished results). Similar studies on the oil-body apolipoproteins of non-cruciferous oilseeds are now in progress in our laboratory.

Reconstitution of oil-bodies. The oil-body membranes and the storage oil could be readily separated from one another by treatment with organic solvents. It was of interest to investigate whether these components could be purified further and then added back together in order to reconstitute the oil-bodies. In the absence of emulsifying agents, such as phospholipids, detergents or apolipoproteins, triacylglycerol droplets coalesced into an amorphous mass, which showed no substructure in the EM. Incubation of purified triolein, or a purified triacylglycerol mixture from rapeseed oil, with delipidated (in diethylether) oil-body membranes resulted in the appearance of stable oil-droplets of similar dimensions to native oil-body membranes, as shown in Fig. 7A. These oil droplets did not have an electron-dense boundary membrane, unlike native oil-bodies, but they were stable to prolonged incubation and were clearly different to simple mixtures of triacylglycerols alone, which spontaneously fused to form one large oil phase. The major oil-body apolipoprotein was a 19 000 polypeptide, which has been purified and extensively studied in our laboratories [10, 17, 18]. The purified 19 000 apolipoprotein was as effective as whole delipidated oil-body membranes in reconstituting oil-droplets of about 1 μm diameter (results not shown). Equally effective was a preparation of phosphatidylcholine liposomes (Fig. 7B), the emulsifier, gum arabic, or various detergents such as Triton X-100 (results not shown).

We conclude that purified triacylglycerols can be reconstituted quite readily into stable oil emulsions by a number of agents, including apolipoproteins, phospholipids, detergents and gum arabic. These agents are all acting as emulsifiers with varying degrees of efficacy. It is pertinent, therefore, to ask why oil-bodies from all of the oilseeds that we and others have examined should be invariably bounded by a proteinaceous membrane, when a phospholipid monolayer alone would serve as an adequate emulsifier. We have previously shown that the oil-body apolipoproteins are synthesized well after the onset of triacylglycerol formation and that they therefore play no part in oil-body formation *per se* [10, 17, 18]. The answer probably lies in the unique resistance of the apolipoprotein-bounded oil-body to attack from destructive agents such as detergents. The oil-bodies must withstand extremes of desiccation, rehydration, heating and

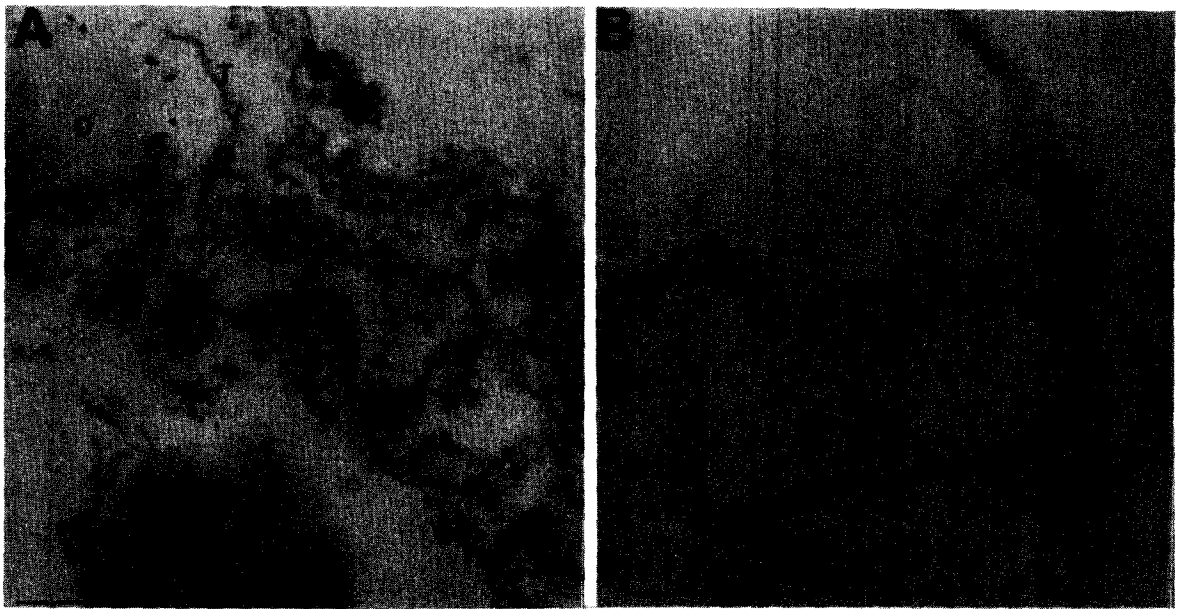

Fig. 5. Electron micrographs of delipidated rapeseed oil-bodies. Oil-body fractions isolated after two floatation stages (OB2) were delipidated (× 3) in 5 vols of (A) chloroform, (B), diethylether. Delipidated membranes were collected following centrifugation at 100 000 g for 1 hr. O, storage oil; T, tubules of oil-body membrane; M, oil-body membrane. Bars represent 1 μm.

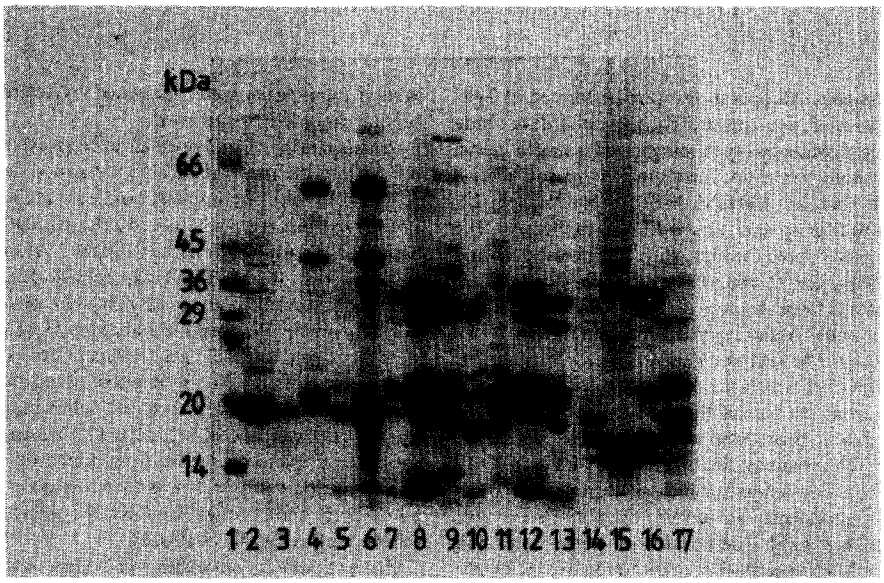

Fig. 6. Polypeptide composition of oil-body apolipoproteins isolated from various species of oilseed. Oil-bodies were isolated following two floatation stages, delipidated (× 3) in 5 vol diethylether, and polypeptides separated on 15% polyacrylamide gels. Lane 1, M_r markers; lane 2, *Brassica napus*; lane 3, *Brassica campestris*; lane 4, *Sinapis alba*; lane 5, *Brassica alboglabra*; lane 6, *Brassica oleracea*; lane 7, *Raphanus sativa*; lane 8, *Arabidopsis thaliana*; lane 9, *Thlapsi arvense*; lane 10, *Matthiola bicornis*; lane 11, *Cochlearia officinalis*; lane 12, *Arabis alpina*; lane 13, *Isalis tinctorius*; lane 14, *Carthamus tinctorius*; lane 15, *Zea mays*; lane 16, *Glycine max*; lane 17, *Helianthus annuus*.

cooling before the storage oil can be mobilized following seed germination. Even then, the storage proteins are first broken down by powerful proteases during the early phases of germination, while the oil-bodies remain intact until a later stage [20]. It is probably necessary that oil-bodies are surrounded by an effective emulsifier which also presents an inert surface during the environmental extremes experienced by dormant and germinating seeds.

EXPERIMENTAL

Chemicals. All solvents were of Analar grade. Biochemicals were obtained from Sigma, unless otherwise indicated.

Plant material. A commercial variety of rapeseed (*Brassica napus* L. cv. Mikado) was used in this study. Rapeseed was harvested from a field crop near Shincliffe, Co. Durham in May–July 1987.

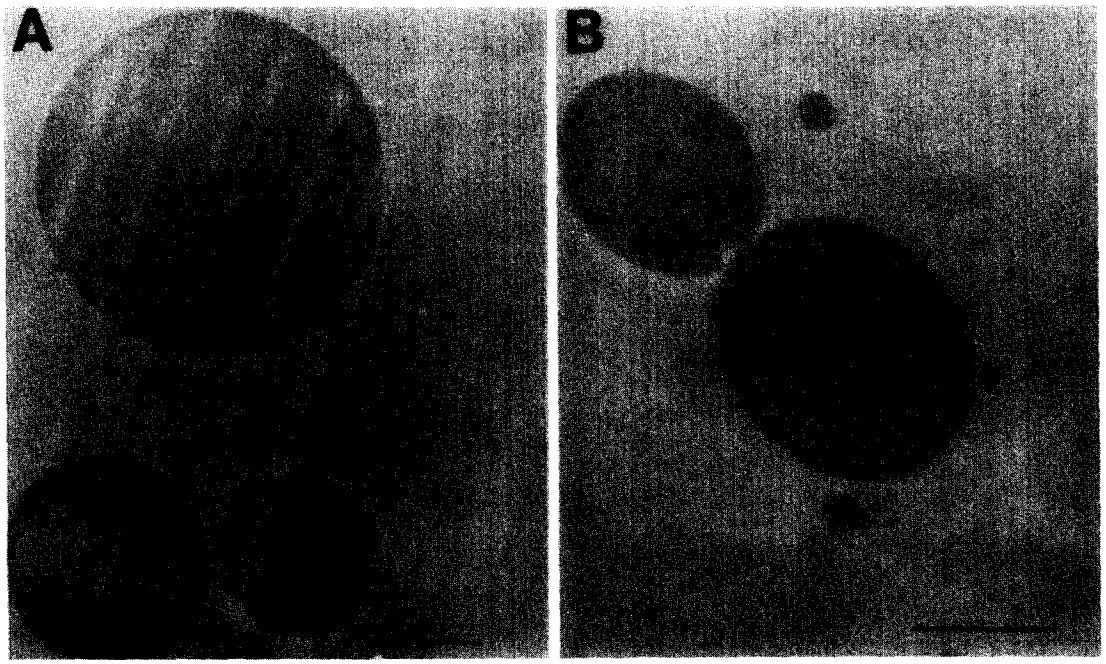

Fig. 7. Electron micrographs of reconstituted oil-bodies. Purified triolein was incubated with (A) purified phospholipid liposomes (100 parts triolein: 2.5 parts phospholipid) or with delipidated rapeseed oil-body membranes (100 parts triolein: 10 parts membrane protein). Mixtures were sonicated for 30 sec, added to 2% LMPagarose, and processed for microscopy. Bars represent 1 μm.

Isolation of oil-bodies. All operations were performed at 0–4°. Embryos were dissected out from freshly harvested developing seeds. The embryos were gently homogenized using a pestle and mortar. The homogenization medium (4 ml/g cotyledons) contained 0.4 M sucrose, 100 mM HEPES–NaOH, pH 7.5, 10 mM KCl, 1 mM $MgCl_2$ and 1 mM EDTA (buffer A). The homogenate was then filtered through 4 layers of chessecloth and centrifuged at 5000 g for 15 min. The crude oil-body fraction (OB_1) was recovered from the top of the 5000 g supernatant, dispersed in 5 vol buffer A and layered beneath a further 20 vol buffer A containing 0.2 M sucrose. This was then centrifuged at 18 000 *g* for 15 min after which the oil-body fraction (OB_2) was again recovered from the top of the gradient. This dispersal/layering/centrifugation procedure was sometimes repeated a further two times. The resultant floating oil-body fraction (OB_4) was essentially free of contamination by soluble proteins or other cell membrane components, as judged by SDS-PAGE and marker enzyme assays [21]. The purified oil-bodies were normally extracted × 3 in 5 vols Et_2O in order to remove triacylglycerols. The resulting membranes were recovered by centrifugation at 100 000 for 1 hr. Oil-bodies were sometimes delipidated in the same way using either $CHCl_3$ or $CHCl_3$–MeOH as solvents.

Lipid analysis. Total lipids were extracted in 20 vols of $CHCl_3$–MeOH (2:1) and aq. contaminants partitioned into the upper (aq.) phase following the addition of 1/5 vol. 0.7% KCl. Upper and lower phases were repeatedly washed with pure lower phase and upper phase solvents respectively [22]. The combined lower phases were dried under a stream of N_2 gas and the residual oil weighed. Alternatively, fatty acid methyl esters were prepared as described previously [23]. The methyl esters were fractionated on a gas chromatograph using a 2 m column packed with 10% DEGS on Chromosorb W, eluted isothermally at 180° using N_2 at 20 ml/min, and detected by flame ionization. Methyl ester peaks were quantified by reference to a methyl heptadecanoate int. standard.

Protein analysis. Total proteins were assayed by the method of ref. [24], in the presence of SDS, by a modified Lowry method [25], or by electrophoresis/Coomassie staining/densitometry. Protein extracts were solubilized prior to SDS–PAGE by incubation at 90° for 5 min in 1.7% SDS, 1% 2-mercaptoethanol, 16% sucrose, 0.1 M Tris–HCl, pH 6.8. Electrophoresis was normally performed using 1.5 mm slab gels containing 15% polyacrylamide. Polypeptide standards of known M_r and mass were also run on each gel. Polypeptides were stained with Coomassie Brilliant Blue R. Relative proportions of the mass in each polypeptide were estimated following scanning of the stained gels with an LKB 2222–010 Ultroscan XL Laser Densitometer. Protein recoveries from whole oil-bodies were relatively low and variable. It was therefore necessary to delipidate all oil-body and homogenate fractions before assaying for protein or performing SDS–PAGE.

Reconstitution of oil-bodies. Oil-bodies were reconstituted by mixing either pure triolein or pure rapeseed oil (neutral oils only) with purified phosphatidylcholine (Sigma P1013) and/or the oil-body membrane apolipoprotein in the proportion 100 parts oil: 10 parts apolipoprotein: 2–5 parts phospholipid (w/w). Mixtures were sonicated for 30 sec and immediately taken for electron microscopy.

Electron microscopy and immunocytochemistry. Oil-body preparations were added to 2% low melting point (LMP) agarose at 60°, vortexed immediately, and cooled with tap water. The agarose-embedded oil-bodies were fixed in 2.5% glutaraldehyde, 1.5% paraformaldehyde, 0.05 M cacodylate buffer, pH 7.0, for 16 hr at 4°. Post-fixation in 1% OsO_4 was for 6 hr. Samples were then dehydrated through a graded alcohol series followed by infiltration and embedding in Spurr's resin. Resin was polymerized at 65° overnight. Gold/silver sections were cut using an

LKB ultrotome 4801A. Sections were sequentially stained with satd aq. uranyl acetate for 15 min and Reynold's lead citrate for 15 min and examined on a Phillips EM400 at an accelerating voltage of 80 kV.

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REFERENCES

1. Miller, C. L. (1988) *J. Am. Oil Chem. Soc.* **65**, 718.
2. Gurr, M. I. (1980) in *The Biochemistry of Plants* Vol. 4. (Stumpf, P. K. and Conn, E. E., eds), p. 205. Academic Press, New York.
3. Stymne, S. and Stobart, A. K. (1987) in *The Biochemistry of Plants* Vol. 9. (Stumpf, P. K. and Conn, E. E., eds), p. 175. Academic Press, New York.
4. Frey-Wyssling, A., Griesehaber, E. and Mühlethaler, K. (1963) *J. Ultrastr. Res.* **8**, 506.
5. Wanner, G., Formanek, H. and Theimer, R. R. (1981) *Planta* **151**, 109.
6. Bair, C. W. and Snyder, H. E. (1980) *J. Am. Oil Chem. Soc.* **57**, 279.
7. Ichihara, K. (1982) *Agric. Biol. Chem.* **46**, 1767.
8. Adams, C. A., Norby, S. W. and Rinne, R. W. (1983) *Crop Sci.* **23**, 757.
9. Huang, A. H. C., Qu, R., Wang, S. M., Vance, V. B., Cao, Y. Z. and Lin, Y. H. (1986) in *Structure and Function of Plant Lipids* (Stumpf, P. K., ed.), p. 239. Plenum, New York.
10. Murphy, D. J. and Cummins, I. (1988) in *Biotechnology for the Fats and Oils Industry* (Applewhite, T. E., ed.). p. 310. Amer. Oil Chem. Soc.
11. Jacks, T. J., Yatsu, L. Y. and Altschul, A. M. (1967) *Plant Physiol.* **42**, 585.
12. Yatsu, L. Y., Jacks, T. J. and Mensarling, T. P. (1971) *Plant Physiol.* **48**, 675.
13. Slack, C. R., Bertand, W. S., Shaw, B. D., Holland, R., Browse, J. and Wright, H. (1980) *Biochem. J.* **190**, 551.
14. Gurr, M. I. and James, A. T. (1984) in *Lipid Biochemistry*, p. 173. Wiley, New York.
15. Breslow, J. L. (1985) *Ann. Rev. Biochem.* **54**, 699.
16. Gurr, M. I., Blades, J., Appleby, R. S., Smith, C. R., Robinson, M. P. and Nichols, B. W. (1974) *Eur. J. Biochem.* **43**, 281.
17. Murphy, D. J., O'Sullivan, J. N. and Cummins, I. (1988) in *The Biological Role of Plant Lipids* (Biacs, P. A., ed.). Plenum, New York (in press).
18. Murphy, D. J. and Cummins, I. (1988) *Plant Sci.* **60**, 47.
19. Bergfeld, R., Hong, Y. N., Kühnl, T. and Schopfer, P. (1978) *Planta* **143**, 297.
20. Murphy, D. J., Cummins, I. and Kang, A. S. (1989) *J. Sci. Food Agric.* **46**, 25.
21. Hills, M. J. and Murphy, D. J. (1988) *Biochem. J.* **249**, 687.
22. Folch, J., Lees, M. and Sloan-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497.
23. Murphy, D. J., Mukherjee, K. D. and Latzko, E. T. (1983) *Biochem. J.* **213**, 249.
24. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 142.
25. Markwell, M. A. K., Haas, S. K., Tolbert, N. E. and Beiber, L. L. (1981) *Meth. Enzymol*, **72**, 269.