# Purification and characterization of oil-bodies (oleosomes) and oil-body boundary proteins (oleosins) from the developing cotyledons of sunflower (*Helianthus annuus* L.)

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Oil-bodies, from the immature cotyledons of sunflower (*Helianthus annuus* L.), were difficult to purify to homogeneity using conventional techniques. The major protein contaminants were albumin and globulin storage proteins. A protocol has been developed, therefore, based upon the stringent washing of the oil-body fraction in 9 M urea, which effectively removed almost all the contaminating protein as judged by SDS/PAGE. The urea-washed oil-bodies were enriched in two major proteins of $M_r$ 19000 and 20000. These proteins were oleosins as demonstrated by their amino acid compositions and the sequence analysis of peptides produced by CNBr cleavage. Far-UV CD spectra of the oleosins in trifluoroethanol, trifluoroethanol/water mixtures and as mixed micelles in SDS, were typical of $\alpha$-helical proteins with $\alpha$-helical contents of some 55 %. The phospholipid content of the urea-washed preparations was less than 0.1 % of that required to form a half-unit membrane surrounding the oil-body. The oil-body surface therefore appears to be an unusual and novel structure, covered largely by an oleosin protein coat or pellicle rather than a conventional fluid membrane, half-unit or otherwise.

## INTRODUCTION

Storage triacylglycerols are deposited in the cells of developing oil seeds in characteristic structures called oil-bodies or oleosomes. These are spherical at early and intermediate stages of seed development, and are slightly greater than 1 $\mu$m in diameter [1], although this may vary between species [2]. The biogenesis and ontogeny of the oil-body has been [3], and still is [4], a contentious area of research. It has been suggested that the oil-body arises by the accumulation of oil between the phospholipid leaflets of the rough endoplasmic reticulum and is then expelled, in some way, to the cytosol [2,5]. On the other hand, nascent oil-bodies have been reported in the cytosol [6,7]. In this context microsomal membranes from developing oil seeds catalysed triacylglycerol formation, and the oil accumulated in discrete droplets in the reaction mixtures [8]. Electron-micrographs indicated that the droplets formed at or on the surface of the vesicles [8]. Isolated oil-bodies are surrounded by a boundary layer containing protein and phospholipid [1], and contradictory reports exist as to the nature of this structure (see [3]). Early studies indicated the presence of characteristic proteins [1,6] and more recent work, particularly by Huang, has identified a unique group of oil-body boundary proteins, the so-called oleosins [2]. The oleosins appear to stabilize the oil-body surface and thus a synchrony in oil and oleosin formation would result in the deposition of the discrete oil-bodies found in oleaceous plant tissue [9]. Murphy and co-workers suggest, however, that oil initially accumulates in the cytoplasm, and only becomes associated with oleosin at later stages of seed maturation [10–12]. Oleosins have received considerable attention in recent years, particularly in relation to oil-body biogenesis and their structural role in stabilizing the triacylglycerol/cytosol oil-body interface. cDNA and genomic clones have been isolated from species such as maize (*Zea mays*), soybean (*Glycine max*), oilseed rape (*Brassica napus*) and sunflower (*Helianthus annuus*) [13–17], demonstrating that they contain regions of high sequence similarity. In particular, all contain a central hydrophobic domain of about 68–74 residues [2], which may form anti-parallel $\alpha$-helices extending into the oil matrix [13], a $\beta$-strand running under the surface of the oil-body [10] or an anti-parallel $\beta$-strand forming a loop into the oil matrix [18]. This domain is flanked by less conserved amphipathic domains at the N-terminus (varying in length from 40 to 60 residues) and the C-terminus (40 residues) which may form helices at the oil-body surface [10,18]. The drawback to these structural models is that they are largely predictive and supported by little characterization of the oleosin protein. This is because the oleosins have proved difficult to purify in a state in which they can be solubilized for detailed structural analysis (see [19]). Similarly, although Li et al. [20] have attempted to avoid these problems by expressing predicted domains in *Escherichia coli*, it is essential that such studies be validated by the analysis of the intact oleosin protein. Previously reported methods of oil-body purification [21] result in a large degree of protein contamination, as judged by SDS/PAGE. Oilseed species contain storage proteins and these are always evident in oil-body preparations. Extensive washing in salt solutions and centrifuging through sucrose gradients results in little clean up. Light microscopy of the oil-body fractions always shows a granular appearance at the boundary of the oil-body and these impurities are impossible to remove even with the detergents Tween 20, Triton X-100, Triton X-114, or CHAPS [22]. Guanidinium chloride, deoxycholate and SDS, on the other hand, remove all the protein and under these conditions the oil-bodies lose integrity and fusion occurs [22]. We have developed, therefore, a novel method for the preparation of largely uncontaminated oil-body fractions from the developing cotyledons of sunflower seed (*Helianthus annuus*) and the purification from these of oleosin protein. This paper describes this procedure and its application to studies of oil-body structure and the physicochemical properties of oleosin.

Abbreviations used: RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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## MATERIALS AND METHODS

### Chemicals and plant material

Sunflower seed (cv. Sunbread 236) was obtained from Twyford Seeds Ltd., Kings Sutton, U.K. All chemicals and solvents were of AnalaR grade.

### Oil-body and oleosin purification

Sunflowers were grown from seed in a 16 h photoperiod at 25 °C and an 8 h night at 18 °C. Seeds were harvested 17–21 days after flowering. The cotyledons were removed and placed in cold 0.1 M potassium phosphate buffer, pH 7.2. All further procedures were carried out at 1–4 °C. Cotyledons were ground in a mortar with 2 parts (w/v) of 0.1 M potassium phosphate buffer, pH 7.2, containing 0.33 M sucrose. Homogenates were filtered through three layers of Miracloth, diluted 5-fold with grinding medium, and centrifuged at 20000 ***g*** for 20 min. Oil-bodies formed a white pad at the surface of the supernatant. After removal, the fat pad was mechanically dispersed in 50 mM Tris/HCl buffer, pH 7.2, containing 9 M urea, at a ratio of 50:1 (v/v) oil to buffer. After stirring at room temperature for 30 min the fat pad was recovered after centrifugation at 20000 ***g*** for 20 min. This was repeated a further three times and the washings recovered on each occasion. Finally the oil-bodies were dispersed in 50 mM Tris/HCl, pH 7.2, at a ratio of 60:1 (v/v) buffer to fat and the fat pad recovered by centrifugation. The preparation was extracted with a 3-fold excess of acetone to remove neutral lipid and the proteins recovered by centrifugation for 2 min at 13500 ***g***. Protein, after two further washes with acetone and diethyl ether, was dried under $N_2$ and stored at −80 °C.

### Light microscopy

Oil-body purity was monitored using light microscopy. Samples were stained with Sudan Red 111 [23] and viewed under Namarski optics.

### Reverse-phase HPLC (RP-HPLC)

The final oil-body protein preparation was dissolved in trifluoroacetic acid (TFA) and diluted with acetonitrile to give an acetonitrile concentration of 80 % (v/v). The proteins were separated by RP-HPLC on either a Lochrosorb C4, C8 or C18 column (25 cm × 0.46 cm) using a linear gradient of water containing 0.07 % (v/v) TFA to acetonitrile containing 0.05 % (v/v) TFA. Flow rate was 1.0 ml/min with detection at 215 nm.

### Gel electrophoresis

SDS/PAGE was carried out using a Tris/Tricine system with a biphasic 10 % (w/v) and 16 % (w/v) polyacrylamide separating gel [24]. Proteins were visualized by staining with Coomassie Brilliant Blue R250.

### Protein determination

Protein was determined by the bicinchoninic acid (BCA) (Pierce Ltd.) method, with incubation for 30 min at 37 °C and BSA as standard.

### CNBr protein cleavage

Excised gel, containing protein, was macerated into an Eppendorf tube with 60 % (v/v) formic acid and one crystal of CNBr [25]. Tubes, wrapped in aluminium foil to reduce CNBr photodegradation, were incubated with shaking for 24 h at room temperature. Samples were diluted with distilled water and after freeze-drying were separated by SDS/PAGE. Peptides were transferred to poly(vinylidene difluoride) (PVDF) membrane (Problott; Applied Biosystems Ltd.) for amino acid sequence analysis [26].

### Amino acid analysis and N-terminal sequencing

Amino acid composition (mol %) and N-terminal amino acid analysis were determined using a Waters Pico-Tag Analyser and an Applied Biosystems 477A Protein Sequencer (Molecular Recognition Centre, Bristol University, Bristol, U.K.) respectively.

### CD spectroscopy

CD spectra were recorded at 20 °C on a JASCO J600 or a Jobin Yvon CD 6 spectropolarimeter. Protein concentrations were determined by weight. Mixed micelles of oleosins in SDS were prepared by the method of Killian et al. [27]. Oleosins were dissolved in trifluoroethanol (TFE); 25 $\mu$l of 1 M SDS was added to 50 $\mu$l of protein solution (10 mg/ml), shaken, then 925 $\mu$l of water was added and the mixture shaken again. This resulted in clear solutions. Blanks were prepared without oleosin. Spectra represent the averages of at least two determinations corrected for baseline and solvent. Deconvolution of the spectra was carried out using the CONTIN programme [28].

### Complex lipid analysis

Oil-bodies were dispersed in 1 ml of 0.1 M potassium phosphate buffer, pH 7.2, and 1 ml of 0.15 M acetic acid was added. Complex lipids and non-esterified fatty acids were extracted by the addition of 3.75 ml of methanol/chloroform (2:1, v/v) and 1.25 ml of chloroform as modified [21] from the method of Bligh and Dyer [29]. The chloroform phase containing the complex lipid was removed and evaporated to dryness under $N_2$. Polar and neutral lipids were purified by TLC on precoated silica-gel plates (silica-gel 60; Merck) with chloroform/methanol/acetic acid/water (170:30:20:7, by vol.) and hexane/diethyl ether/acetic acid (70:30:1, by vol.) respectively. Lipid areas, located by lightly staining with $I_2$ vapour, were removed from the plates and the esterified fatty acids transmethylated with methanolic HCl [30]. Fatty acid methyl esters were analysed by GLC and quantified using methylheptadecanoic acid as an internal standard. Complex-lipid quantification was based upon fatty acid contents [21]. Analyses were carried out on oil-bodies prepared on three separate occasions and from different batches of seed. Results were similar in each case.

Phosphatidylethanolamine and phosphatidylserine were also located on the TLC plates using ninhydrin reagent [31].

## RESULTS

### Oil-body purification

Isolated oil-bodies, sequentially treated with 9 M urea in Tris/HCl buffer (pH 7.2), were almost free of contaminating material, as judged by light microscopy, and had a smooth surface without the usual granular appearance. They were also of a similar diameter (1–2 $\mu$m) to those present *in vivo* in developing seed cotyledons.

Purification resulted in the progressive removal of contaminating proteins (Figure 1). The 'crude' oil-body fraction (Figure 1, track 3) contained numerous proteins which were poorly resolved by SDS/PAGE. After washing the oil-bodies in KCl, much background material was removed, producing a number of

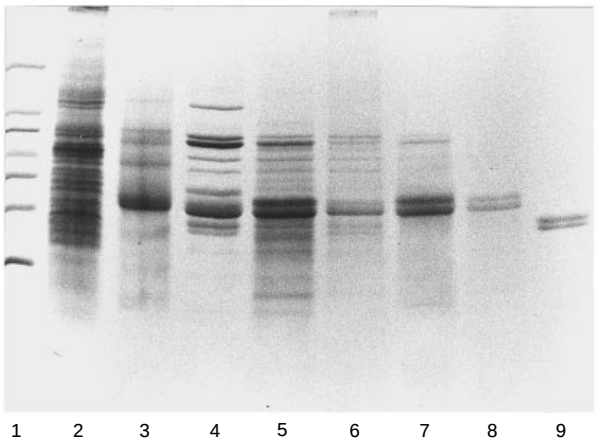


**Figure 1 SDS/PAGE of oleosin purification**

Track 1, relative molecular mass markers, in decreasing $M_r$, 66000, 45000, 36000, 29000, 24000, 20100 and 14200; track 2, total protein from sunflower microsomes; track 3, crude oil-body total protein; track 4, KCl-washed oil-body total protein; tracks 5–8, supernatants from sequential washes with 9 M urea; track 9, total protein (oleosins) from urea-washed oil-bodies.

**Table 1 Amino acid compositions (mol%) of oleosins, from cDNA sequences [16,17] and purified from oil-bodies ($M_r$ 19000 and 20000)**

Abbreviation: nd, not determined.

| Amino acid | Cummins and Murphy [16] | Thoyts et al. [17] | 19000-$M_r$ protein | 20000-$M_r$ protein |
|---|---|---|---|---|
| Asn + Asp | 5.5 | 4.9 | 5.9 | 5.3 |
| Gln + Glu | 9.4 | 10.4 | 8.3 | 8.8 |
| Ser | 7.2 | 4.4 | 6.5 | 6.1 |
| Gly | 12.8 | 14.8 | 18.2 | 21.5 |
| His | 2.8 | 4.4 | 2.0 | 2.2 |
| Arg | 4.4 | 3.9 | 4.3 | 4.1 |
| Thr | 12.2 | 11.5 | 9.6 | 9.0 |
| Ala | 7.8 | 3.9 | 7.1 | 8.8 |
| Pro | 3.3 | 4.9 | 5.0 | 5.1 |
| Tyr | 3.3 | 2.7 | 2.5 | 2.3 |
| Val | 6.1 | 6.6 | 6.6 | 5.6 |
| Met | 1.1 | 3.9 | 0.8 | 0.6 |
| Cys | 0.0 | 0.0 | 0.0 | 0.0 |
| Ile | 5.5 | 6.0 | 3.3 | 2.5 |
| Leu | 10.5 | 9.3 | 12.1 | 11.0 |
| Phe | 4.4 | 3.3 | 3.3 | 2.6 |
| Lys | 3.3 | 4.9 | 4.5 | 3.9 |
| Trp | 0.0 | 0.0 | nd | nd |

well-defined protein components (Figure 1, track 4). The major proteins in the salt-washed oil-bodies were N-terminally sequenced and most corresponded to previously characterized storage albumins and globulins. Two proteins, however, of $M_r$ 19000 and 20000, were N-terminally blocked. Sequential washing of the oil-bodies with 9 M urea removed the storage protein contaminants (Figure 1, tracks 5 to 8) and after four washes only the two N-terminally blocked proteins were present and in almost equal amounts (Figure 1, track 9). This simple and rapid protocol, therefore, gave highly purified oil-bodies which contained only two proteins with similar $M_r$ to the oleosins characterized from other sources [4].

## Oleosin characterization

Attempts to separate these two components by RP-HPLC on C4, C8 or C18 columns were unsuccessful, the proteins always co-eluting in one peak [22]. Purified oil-body protein was, therefore, separated by semi-preparative SDS/PAGE and the two proteins blotted on to ProBlott for amino acid analysis. The results (Table 1) show that the proteins had almost identical amino acid compositions. Amino acid sequences, deduced from cDNA sequences, are available for two sunflower oleosins: a partial 19500-$M_r$ protein [16] and a full-length 20500-$M_r$ protein [17]. The deduced amino acid compositions of these oleosins were similar to those of the purified proteins, with the exception of the glycine contents which were lower in the compositions calculated from cDNA sequences. The reason for this is not known, as glycine buffers were not used during the purification procedure. Since the two purified oil-body proteins were N-terminally blocked, CNBr digestion was used to generate peptides for sequencing. N-terminal sequences of the major peptides obtained from the 19000-$M_r$ and 20000-$M_r$ proteins were similar (Table 2) to each other and with sequences encoded by the oleosin cDNAs [16,17].

Because of the similar properties and composition of the two proteins, the oleosin mixture was used in physicochemical studies. The protein preparation was subject to RP-HPLC to remove contaminants not revealed by SDS/PAGE. Far-UV CD spectra of the oleosin in TFE and TFE/water mixtures were typical of an $\alpha$-helical-rich protein with negative maxima around 208 and 222 nm and a positive maximum at 190 nm (Figure 2). In TFE/water, the $\alpha$-helical content decreased with increasing water concentration. The CD spectrum in SDS micelles showed a similar $\alpha$-helical-rich spectrum (Figure 2). The shift to higher wavelength in the SDS micelles is most probably due to solvation differences between SDS and the TFE and TFE/water mixtures. Deconvolution of the spectrum in TFE and in SDS micelles indicated an $\alpha$-helical content of some 55–60% and 50% respectively.

## Oil-body lipid composition

The proportions of neutral and polar lipids in freshly prepared oil-body preparations, and in a microsomal membrane fraction from the same tissue, are given in Table 3. The major neutral lipid was triacylglycerol with only a small proportion of diacylglycerol. However, with progressive purification of the oil-bodies by urea washing, the proportion of triacylglycerol increased with a corresponding decrease in polar lipid. Little unesterified fatty acid was evident. Major phospholipids were phosphatidylcholine and phosphatidylethanolamine with smaller quantities of phos-

**Table 2 N-terminal sequences of CNBr-cleaved peptides derived from oleosins of $M_r$ 19000 and 20000 and comparison with published sequences**

| Oleosin | Peptide $M_r$ | Sequence |
|---|---|---|
| Cleaved | | |
| 20000 | 10000 | **I**R**LAV**A**GF**LRRSDH |
| 20000 | 9200 | WR**LAV**X**GF**IHRET |
| 20000 | 8200 | **I**R**LAV**A**GFL**NNGAFR |
| 19000 | 12000 | **I**R**LAV**A**GFL**THXXT |
| 19000 | 9500 | **I**R**LAV**A**GFL**TEG |
| Published | | |
| 19500 [16] | Residues 85–105 | **I**G**LAV**T**GFL**ASGTFGLTGLSSL |
| 20500 [17] | Residues 92–112 | **I**G**LAV**T**GFL**TSGTFGLTGLSSL |

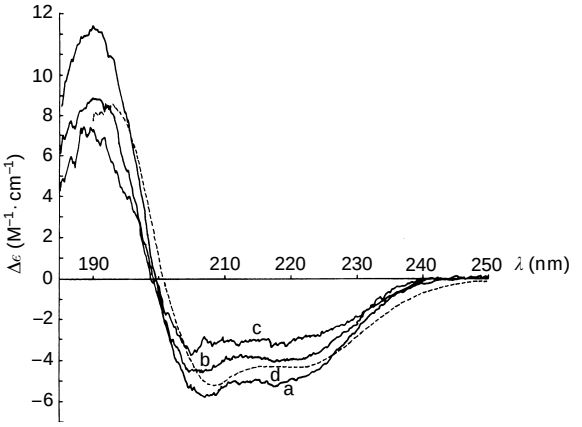


**Figure 2 CD spectra of oleosins in (a) 100% TFE, (b) 75% (v/v) aq. TFE, (c) 50% (v/v) aq. TFE and (d) oleosins in SDS-micelles**

Concentrations: lines a, b and c were produced at a concentration of 0.1 mg/ml and line d at 1 mg/ml. Temperature was 20 °C.

**Table 3 Lipid compositions of oil-bodies (%)**

Abbreviations: TAG, triacylglycerol; DAG, diacylglycerol; SE, sterol ester; NEFA, non-esterified fatty acid; nd, not detectable. The results are the means of two determinations ± S.E.M.

| Sample | TAG | DAG | SE | NEFA | Polar |
|---|---|---|---|---|---|
| Sunflower KCl-washed oil-body | 98 ± 2 | 0.8 ± 0.3 | nd | 0.2 ± 0.1 | 0.8 ± 0.2 |
| Sunflower crude oil-body | 96 ± 3 | 1.0 ± 0.1 | 0.1 ± 0.02 | nd | 2.5 ± 0.4 |
| Sunflower urea-washed oil-body | 98 ± 2 | 0.8 ± 0.1 | 0.1 ± 0.02 | nd | 0.7 ± 0.1 |
| Sunflower microsome | 36 ± 3 | 4.6 ± 1 | 0.8 ± 0.02 | nd | 58.1 ± 4 |

**Table 4 Sunflower polar lipid composition (%)**

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; nd, not detected. The results are the means of two determinations ± S.E.M.

| Sample | PC | PE | PI | PS |
|---|---|---|---|---|
| Crude oil-body | 81 ± 3 | 13 ± 2 | 5 ± 1 | nd |
| Urea-washed oil-body | 79 ± 4 | 13 ± 2 | 8 ± 1 | nd |
| Microsome | 76 ± 3 | 12 ± 1 | 10 ± 2 | 2 ± 0.2 |

phatidylinositol (Table 4). Little or no phosphatidylserine was observed. No phosphatidylserine was found in purified oil-bodies from other oil seeds [safflower (*Carthamus tinctorius*) and maize (*Zea mays*)] [22]. Phosphatidic acid and unesterified fatty acids were only evident in oil-body fractions after storage at −80 °C. Fatty acid compositions of the total polar lipid from crude and urea-purified oil-body fractions and microsomal membrane preparations were similar, the major fatty acids being oleic and linoleic acid (Table 5).

In view of the lipid and protein contamination associated with most oil-body preparations, it was considered important to determine the phospholipid and oleosin contents of oil-bodies purified by urea washing, and to assess their contributions to the oil-body boundary layer. Because these oil-bodies consist only of lipid and oleosins of known mass, it is possible to calculate

**Table 5 Fatty acid composition of the polar lipids (%)**

Abbreviations: TP, total polar lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine, PI, phosphatidylinositol; PS, phosphatidylserine; nd, not detected. The results are the means of two determinations ± S.E.M.

| | | Fatty acid (%) | | | |
|---|---|---|---|---|---|
| Sample | Lipid | 16:0 | 18:0 | 18:1 | 18:2 |
| Sunflower crude oil-body | TP | 8 ± 1 | 7 ± 1 | 56 ± 3 | 29 ± 2 |
| | PC | 5 ± 1 | 6 ± 1 | 64 ± 4 | 24 ± 2 |
| | PE | 17 ± 2 | 5 ± 1 | 43 ± 3 | 35 ± 3 |
| | PI | 21 ± 2 | 14 ± 1 | 35 ± 2 | 29 ± 2 |
| Sunflower urea-washed oil-body | TP | 9 ± 1 | 9 ± 1 | 54 ± 3 | 28 ± 2 |
| | PC | 6 ± 1 | 2 ± 1 | 74 ± 3 | 19 ± 2 |
| | PE | 19 ± 2 | 6 ± 1 | 43 ± 2 | 33 ± 2 |
| | PI | 26 ± 2 | 6 ± 1 | 54 ± 3 | 14 ± 2 |
| Sunflower microsomes | TP | 12 ± 1 | 8 ± 1 | 50 ± 3 | 30 ± 2 |
| | PC | 8 ± 1 | 11 ± 1 | 60 ± 4 | 20 ± 2 |
| | PE | 22 ± 1 | 3 ± 1 | 40 ± 2 | 35 ± 3 |
| | PI | 22 ± 1 | 19 ± 2 | 33 ± 2 | 26 ± 2 |
| | PS | 19 ± 1 | nd | 48 ± 2 | 33 ± 2 |

protein/lipid molar ratios with some accuracy. The oleosin:phospholipid ratio was always close to 1:4 with the oleosins accounting for 4–4.2% by weight of the total oil-body. It is also possible to calculate the amount of phospholipid required to form a half-unit membrane around an oil-body. Calculations, based on constants and methods given by Tzen and Huang [32], show that the urea-purified oil-bodies with diameters of 1 and 2 $\mu$m have phospholipid contents of $3.46 \times 10^{-19}$ g and $2.77 \times 10^{-18}$ g respectively. This is only 0.004% and 0.009%, respectively, of that required to occupy the volume of a half-unit membrane 2.5 nm thick. Estimates, based on polar lipid head-group area, give values of less than 0.01% of the phospholipid required to cover the surface area of an oil-body. The phospholipid content of purified oil-bodies from the developing cotyledons of safflower was also much too low to account for a conventional half-unit membrane structure [22].

## DISCUSSION

Oil-bodies were difficult to purify to homogeneity using conventional methods. The major contaminating proteins were albumin and globulin storage proteins. Stringent washing of the oil-bodies in 9 M urea, however, removed almost all the non-oil-body protein to yield stable, spherical oil-bodies with a smooth surface and of a similar size to those found *in vivo*. Why the urea treatment denatures the contaminating proteins leaving the oleosins intact is unclear. Certainly the oleosins are not stabilized by phospholipids since these can be completely removed by phospholipase treatment with no effect on the integrity of the oil-body or the solubility of the oleosins in urea [22]. In this respect it is interesting that oleosins and storage proteins were completely solubilized in oil-bodies treated with guanidinium chloride, deoxycholate and SDS [22].

The N-terminally blocked proteins ($M_r$ values of 19000 and 20000) in the purified oil-bodies yielded similar CNBr cleavage products and these had similar sequences to those deduced from oleosin cDNA clones [16,17]. The oleosin was soluble in TFA and TFE after removal of oil-body lipid and without the need for detergent treatments. Deconvolution of the CD spectra in TFE indicated an $\alpha$-helical content of some 55% and this decreased

with the addition of water. TFE is widely used as a structure-inducing solvent with a low dielectric-constant which strengthens intramolecular bonds and stabilizes secondary structures [33]. Although there is evidence for a TFE induction of helical regions in proteins [34,35], it is considered [36] that it is not a helix-inducing solvent in the sense that it brings about helix formation independently of sequence, but rather that it stabilizes regions with the propensity to form helices. Our CD analyses contrast with studies of oilseed rape oleosins which had 11–16 % $\alpha$-helix and 58–73 % $\beta$-sheet [19]. Solid-state Fourier transform infrared of the protein also indicated a low $\alpha$-helix content and a higher level of $\beta$-sheet [19]. Unfortunately, these reports gave no details of solvent or concentration and it is evident that the addition of water to TFE brings about a significant decrease in the $\alpha$-helical content of the protein (Figure 2). It is also noteworthy that the CD spectra of oleosin and SDS in mixed micelles were similar to those in TFE (Figure 2). Oleosins are hydrophobic and their conformations in mixed micelles, and in TFE, may more accurately reflect their structure at the oil-body surface. The high levels of $\alpha$-helix in the present study are not consistent with the models of Huang and colleagues [18] or Murphy et al. [10] which propose that oleosins interact with triacylglycerols by $\beta$-sheet structures. Some re-evaluation is clearly required.

The oleosin contributed some 4 % to the total mass of the oil-body and this was similar to oilseed rape and maize [4], but disagreed with the study of Murphy which reported values greater than 12 % [12]. The protein:phospholipid molar ratios were 1:4, compared with 1:13 [32]. The urea-washed oil-bodies, therefore, have a much lower phospholipid content than those produced by other methods. Although phospholipid was removed during urea washing, the oil-bodies remained stable and of similar size. In fact phospholipase treatments of the oil-body preparation had no effect on stability, whereas protease digestion brought about the rapid breakdown of oil-body integrity [22]. The phospholipid present in the urea preparations was less than 0.1 % of that required to form even a half-unit membrane around the oil-body. Similar results were reported by Kleinig et al. [37] studying oil-bodies from carrot (*Daucus carota*), and Harwood et al. [38] failed to detect a membrane, half-unit or otherwise, surrounding castor bean (*Ricinus communis*) oil-bodies using electron microscopy. In contrast, the phospholipid content reported in [32] was just sufficient to form an oil-body delimited by a half-unit membrane. The oil-body surface, therefore, appears to be an unusual structure covered largely by an oleosin protein coat or pellicle rather than a conventional membrane. Other differences exist between urea-washed oil-bodies and those obtained by other methods. Huang [2] reported high levels of phosphatidylserine and implicated this negatively charged phospholipid in oil-body boundary structure. No phosphatidylserine, or other negatively charged lipids, were found either in the urea-purified oil-bodies or in oil-body fractions given a salt wash [22].

Oleosin clearly plays the major role in oil-body stabilization with little structural contribution from phospholipid. The negatively charged oil-body surface [18] appears to be an inherent property of oleosin (and possibly its interactions with triacylglycerol) rather than being influenced by phospholipid. This, together with the high $\alpha$-helical content, may indicate that more of the oleosin partitions above the oil-body surface than previously thought. The apparent lack of a half-unit membrane has implications for oil-body biogenesis and supports the suggestion that triacylglycerol may arise at or on the surface of the endoplasmic reticulum [3], where it becomes associated and stabilized with oleosin; the synchrony in oil and oleosin production governing the final size of the oil-body.

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