Secondary structure of oleosins in oil bodies isolated from seeds of safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.)

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Oil bodies were isolated from mature seeds of sunflower (*Helianthus annuus* L.) and safflower (*Carthamus tinctorius* L.). Oil body preparations containing only oleosin proteins could be obtained from safflower seeds by salt-washing followed by centrifugation on discontinuous sucrose density gradients. However, it was necessary to treat sunflower oil bodies with urea to obtain preparations of similar purity. Incubation of the oil bodies with proteinases gave two fragments with molecular masses of 6 and 8 kDa which were protected from digestion. These fragments represented the hydrophobic domain of the oleosins, as determined by N-terminal sequencing. Intact and proteinase-treated oil bodies of both species were analysed by Fourier-transform infrared spectroscopy, as dry films and in aqueous medium, the spectra being compared with those obtained for pure oil samples in order to identify the bands resulting from the oleosin proteins and protected peptides. This investigation showed that the hydrophobic domain of the oleosins in intact oil bodies is predominantly α-helical in structure and that the conformation was not greatly affected by washing the oil bodies with urea during preparation.

INTRODUCTION

Seeds of many plant species store triacylglycerols (TAGs) in discrete organelles called oleosomes or oil bodies. These unusual organelles are about 0.6–2 μm in diameter [1] and contain a core of TAGs surrounded by an outer coat, thought to consist of a phospholipid (PL) monolayer and a single class of proteins termed oleosins [2]. The mechanisms of oil body biogenesis and the role of the oleosins in this process are, as yet, unclear [3]. Oleosins have molecular masses of about 15–26 kDa and are postulated to stabilize the oil body by preventing coalescence, particularly during seed desiccation [4]. They may also act as a binding site for lipases during the mobilization of reserves which occurs during seed germination [4]. A number of cDNA and genomic clones encoding seed oleosins have been isolated from species, including maize (*Zea mays*), rape (*Brassica napus*), *Arabidopsis thaliana* and sunflower (*Helianthus annuus*) [5–9]. The amino acid sequences of all the oleosins so far studied can be divided into three distinct structural domains: N-terminal amphipathic, central hydrophobic and C-terminal amphipathic [1,10,11]. The hydrophobic domain consists of approx. 70 amino acids and its sequence is highly conserved between species. It is therefore likely to be essential for oleosin function and is thought to be inserted into the hydrophobic core of the oil body. The N-terminal (50–70 amino acids) and C-terminal (55–98 amino acids) domains, however, are much less conserved in their amino acid sequences and have been suggested to lie on the surface of the oil body, with positively charged residues orientated towards the oil body and negatively charged residues facing the cytosol. The charge properties on the oil body surface may therefore explain why oil bodies do not coalesce [12,13].

There has been considerable interest in the oleosins and their modes of interaction with TAGs. In particular, it has been suggested that the hydrophobic central domain forms a 'hairpin-like' structure, consisting of two antiparallel β-sheets connected by a proline-rich turn (or 'proline knot') which is buried in the TAG matrix of the oil body. This model is supported by secondary structure predictions using standard algorithms, although these are based on soluble globular proteins rather than hydrophobic proteins, such as oleosins [1,14]. Preliminary CD and solid-state infrared spectroscopy studies of oleosins isolated from peanut [15] and oil seed rape [11] were also consistent with this predicted secondary structure. Jacks et al. [15] reported high levels of β-sheet using the CD of proteins dissolved in ethanol/acetic acid/water and the IR spectra of dry specimins in KBr discs. Similarly, Li et al. [11] reporting 40–50% β-sheet and 12–17% α-helix structure in oleosin protein isolated from oil seed rape using Fourier transform infrared spectroscopy (FTIR) in KBr discs and CD, but details of solvents and conditions used for the CD were not provided. Li et al. [11] also observed that, under high-salt conditions, the oleosins readily formed dimers, with little change in the protein secondary structure [15]. This observation led to the suggestion that, rather than forming antiparallel β-sheets within a single molecule, the central β-strand of the oleosins may form dimeric or oligomeric associations, allowing this region of the oleosin molecule to penetrate deeply into the matrix of TAG [15]. The N- and C-terminal domains of oleosins have been predicted to contain amphipathic α-helix and some random-coil structure [11], and this has been supported by CD and FTIR spectroscopy of a part of the N-terminal domain of a sunflower oleosin expressed in *Escherichia coli* [16]. When this domain was reconstituted into liposomes, it was estimated to contain about 20% α-helical structure and 30–40% β-strand structure, with the rest being mainly random coil.

Millichip et al. [17] have recently re-investigated the secondary structure of oleosins purified from sunflower, by determining CD and FTIR spectra in trifluoroethanol, trifluoroethanol–water and

Abbreviations used: ATR, attenuated total reflectance; PL, phospholipid; TAG, triacylglycerol; FTIR, Fourier-transform infrared.

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as mixed micelles in SDS. In contrast to the previous studies, the spectra obtained were typical of a protein rich in α-helix, which was calculated to account for about 55 % of the structure [17]. The validity of these results has since been questioned by Ratnayake and Huang [18], based on the harsh conditions (treatment with 9 M urea) used to purify the oleosins. Some unfolding would certainly occur during urea treatment, but the major secondary structure elements would be expected to reform upon removal of the denaturant. A more serious criticism of these and all previous studies of the structures of isolated oleosins relates to the removal of the protein from the environment of the oil body. In particular, removal of the central domain from the highly hydrophobic environment of the TAG matrix could result in destabilization of the native secondary structure We have therefore used FTIR spectroscopy to determine the secondary structures of oleosins present in intact oil body preparations from sunflower and safflower, the latter species being selected because oil bodies containing only oleosin proteins could be isolated by mild purification procedures. In addition, the effects of urea on the secondary structures of oleosins present in the oil body preparations were determined.

MATERIALS AND METHODS

Materials

Sunflower (*Helianthus annuus* L., cv. Sunbred 246) and Safflower (*Carthamus tinctorius* L., cv. Gila) plants were grown in a glasshouse with a photoperiod of 16 h light at 25 °C and 8 h dark at 18 °C. Mature seeds were harvested randomly from minimum populations of ten plants. All chemicals were purchased from Sigma Chemical Company (Poole, Dorset, U.K.) or BDH Chemicals (Poole, Dorset, UK). Solvents were from BDH Chemicals or Rhône Poulenc (Manchester, U.K.).

Oil body purification

Approximately 5 g of cotyledons were isolated from mature sunflower and safflower seeds, the safflower seeds being initially soaked overnight at room temperature. The oil bodies were initially isolated and washed three times, based on methods published elsewhere [19]. The embryos were homogenized using a Polytron probe (Kinematica, Lucerne, Switzerland) in 10 ml of 50 mM Tris/HCl (pH 7.5) containing 500 mM sucrose, 0.5 mM EGTA, 0.5 mM EDTA and 2 M KCl. The homogenate was filtered through two layers of Miracloth (Calbiochem, Nottingham, U.K.), transferred to 30 ml Corex tubes and overlaid consecutively with two 5 ml layers of buffer containing 250 and 125 mM sucrose respectively, but otherwise identical with the above buffer. The oil bodies were recovered by centrifugation at 5000 ***g*** for 20 min using an FO650 fixed-angle rotor (Beckman, High Wycombe, U.K.). The oil body pad was removed from the top of the sucrose density gradients and homogenized, using a glass homogeniser, in 10 ml of the 500 mM sucrose buffer. The above process was then repeated three times. The oil bodies were then washed similarly four more times, except that the 2 M KCl was omitted and the gradients were made in 13 ml ultracentrifuge tubes, consisting of three 4 ml layers of the 500, 250 and 125 mM sucrose buffers, respectively. The oil bodies were recovered by ultracentrifugation at 100000 ***g*** for 20 min using a TST 41.14 swing-out rotor (Kontron Instruments, Watford, U.K.), resuspended in 10 mM Tris/HCl (pH 7.5) and subjected to a final ultracentrifugation step before being resuspended in 10 vol. of 10 mM Tris/HCl (pH 7.5). Alternatively, the oil bodies were resuspended in 8 M urea/50 mM Tris/HCl (pH 7.5) and incubated at room temperature, with shaking, for 30 min. The oil bodies were then recovered by ultracentrifugation and rewashed in the urea buffer before being resuspended in 10 mM Tris/HCl (pH 7.5) as described above.

PAGE

Proteins and peptides were separated by SDS/PAGE in 0.75 mm biphasic slab gels consisting of a 10 % (w/v) polyacrylamide stacking gel and a 16 % (w/v) polyacrylamide separating gel using the Tris/Tricine system [20]. Before resolution of the oil body proteins by SDS/PAGE, the oil bodies were delipidated by three washes in diethyl ether. Proteins were visualized by staining with Coomassie BB-R250 (Sigma).

Proteinase digestion of oil bodies

Proteinase digestion was performed on salt-washed oil bodies, unless stated otherwise in the Figure legends. Oil bodies were removed from the 10 mM Tris/HCl (pH 7.5) buffer by ultracentrifugation, as described above, and resuspended in an equal volume of a buffer containing 10 mM Tris/HCl (pH 8.25) and 1 mM $CaCl_2$. Portions of 800 μl of the oil body suspension were incubated, as described in the Figure legends, with 8.8 units of thermolysin from *Bacillus thermoprateolyticus rokko* (Sigma) or 2.8 units of proteinase K from *Tritirachium album* (Sigma) in a total volume of 1 ml, and incubated at room temperature, with shaking, for 4 h. The proteinase was then inhibited by the addition of 200 μM PMSF and the oil bodies were removed from the reaction mixture by microcentrifugation for 10 min. The oil bodies were then purified from any enzyme contamination by washing four times using the method of Slack et al. [19].

N-terminal sequencing

Proteins and peptides separated by SDS/PAGE were transferred to a PVDF membrane (Applied Biosystems, Foster City, CA, U.S.A.) and stained according to the manufacturer's instructions, except that the gels were pre-run for 1 h in 0.1 mM sodium thioglycolate and acetic acid was omitted from the destaining solution. The bands of interest were then excised and N-terminally sequenced.

FTIR spectroscopy

The FTIR spectra were recorded on an FTS 6000 spectrometer (Bio-Rad, Watford, U.K.) equipped with a HgCdTe detector. The samples were placed in a MicroCircle liquid attenuated total reflectance (ATR) cell (SpectraTech, Warrington, U.K.) with a ZnSe crystal. For each spectrum 256 scans at 2 cm^{-1} resolution were co-added. The empty cell was used as reference. First, the spectra of water, 10 mM Tris/HCI (pH 7.5) buffer solution, and of pure safflower or sunflower oil were recorded separately. Then a 150 μl emulsion of oil bodies in 10 mM Tris/HCl (pH 7.5) was injected into the ATR cell. Within 20 min there was a visible deposition of oil bodies on the ZnSe crystal, shown by the appearance of lipid bands in the IR spectrum. From the solution spectra, first the Tris buffer spectrum and then the oil spectrum (safflower or sunflower) were digitally subtracted. However, this two-stage subtraction was quite difficult, presumably because of the large variability in the optical properties (scattering and refractive index) in this system. The low intensity and the potentially large subtraction artefacts made the resulting spectra unsuitable for a detailed structure analysis.

The cell was therefore drained, the deposited oil bodies on the crystal washed with water to remove all buffer salts, and then

Table 1 Conformational assignments of amide I, II and III infrared bands (cm^{-1})

The conformational assignments were performed according to previous studies [21–25], the band positions are given to ± 2 cm^{-1}. (sh) denotes shoulders in the FTIR spectrum.

Amide I		Amide II		Amide III	
1690	β-sheet and turns	1568	Turns	1300	Turns
1680	β-sheet	1545	α-helix	1280-1260	α-helix
1672	Turns	1524	Antiparallel β-sheet	1245-1220	β-sheet
1656	α-helix	1517	β-sheet, α-helix (sh)		
1650	Random				
1636	β-sheet				
1632	Antiparallel β-sheet				
1625	Intermolecular β-sheet				

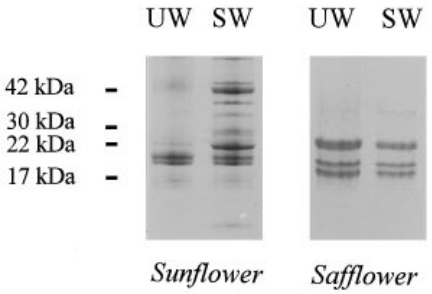

Figure 1 Protein profile of oil body preparations

Protein and peptide compositions of oil body preparations after urea-washing (UW) and salt-washing (SW). The positions of molecular-mass markers are shown on the left.

dried with a stream of dry air. The spectrum was recorded and the strong lipid bands were partially removed by subtraction of the safflower oil spectrum. Complete removal was not possible and some distortions remained in the place of the strongest bands (e.g. the 1738 cm^{-1} band in the spectra). However, these did not affect the amide I and II bands of the protein spectrum. To confirm the assignment of α-helical structure in the protected fragments of both plant species, the amide III band was investigated after washing the dried films with dry ethanol to remove the oil phase, which has an overlapping absorption in this region (in particular a sharp band at 1237 cm^{-1} that could be mistaken for β-sheet if not compensated for properly). A broad band was observed in the 1250–1300 cm^{-1} region, which is consistent with the high content of α-helix (results not shown). Although alcohols can denature proteins and induce α-helical structures, the unchanged shape of the amide I band indicated that this did not occur in the present study.

Most of the results were obtained using dry oil body films because of the higher quality of the spectra and the greatly reduced chance of subtraction artefacts. However, to confirm the validity of this approach, some samples were also measured in aqueous medium. For this the ATR cell was filled with distilled water, which was then gradually replaced with a sample of oil bodies. The spectrum of the deposited oil bodies was obtained by subtraction of the initial water spectrum. Pure water was used to avoid effects of buffer salts but, according to the IR spectra, this treatment had very little influence on the amide bands of the protein, compared with the original buffer emulsion.

In order to obtain a better estimate of the secondary structures, the amide region of the spectra was Fourier-deconvoluted using the method of Griffiths and Pariente [21] with $\gamma = 3.5$ and $\times = 0.2$. The amide I band was then fitted with a sum of Gaussian bands using a spectrometer software routine based on the Levenberg–Marquardt algorithm [22]. The conformation assignments of amide bands were performed according to previous studies [23–27]; the band positions are given to ± 2 cm^{-1} (Table 1).

RESULTS

Treatment with urea is necessary to remove non-oleosin proteins to give sunflower oil bodies containing only oleosins [17], whereas 'pure' oil bodies (i.e. containing only oleosin proteins) can be prepared from safflower by separation on discontinuous sucrose density gradients [19]. The protein compositions of safflower and sunflower oil bodies, purified by different protocols, are compared by SDS/PAGE in Figure 1. Safflower oil bodies prepared by salt-washing and separation on sucrose density gradients (salt-washed oil bodies) contained five main components of 26.6 kDa, 25.7 kDa, 21.6 kDa, 19.3 kDa and 18.6 kDa, although the calculation of the molecular masses varies slightly from a previous study of this species [19]. In contrast, the sunflower oil bodies prepared by this procedure contained numerous proteins and peptides and it was not possible to identify the oleosins (Figure 1). Urea treatment had little effect on the protein composition of safflower oil bodies, which suggests that the treatment did not disrupt the oil body structure. The protein composition of sunflower oil bodies was, however, dramatically affected by urea washing, which left only two major bands of 20.6 kDa and 19.5 kDa, corresponding to the sunflower oleosins, with a third minor band of $\sim$ 17.5 kDa being observed in overloaded samples. Whereas Millichip et al. [17] showed that sunflower oleosins were present in the supernatant after urea washing, in the present study they remained completely in the oil bodies (results not shown). It is likely that, in the previous study, the oil bodies were not completely removed from the supernatant after urea washing [17]. The observation that treatment with urea did not disrupt the protein composition of safflower oil bodies indicates that it is a valid method for the purification of sunflower oil bodies containing only oleosin proteins.

Digestion with proteinase has been used previously to identify fragments of oleosins which are proteolytically protected [2] and hence presumably buried in the core of the oil bodies. Proteolytic digestion of salt-washed safflower and sunflower oil bodies with thermolysin or proteinase K gave two fragments with molecular masses of approx. 6 and 8 kDa (Figure 2), which is in general agreement with the size of the trypsin-protected fragment obtained for maize oil bodies [2]. It seems likely, considering that the hydrophobic domain of the oleosins is highly conserved, that the two fragments result from incomplete digestion of the oleosins rather than from separate oleosin isoforms. The 8 kDa protected fragment from safflower was purified from digested oil bodies and subjected to N-terminal sequencing. The first five residues (Leu-Ala-Gly-Gly-Ser) could be clearly aligned with sequences present at the N-terminal end of the hydrophobic domains of oleosins from other species (Figure 3), confirming the identity of the protein as oleosin and the site of cleavage. Based on this cleavage site and the sequences shown in Figure 3, it can be calculated that the hydrophobic central domain of the oleosins is approx. 8 kDa, which is in good agreement with the larger protected fragments prepared from sunflower and safflower. Further evidence that the protected fragments represent the

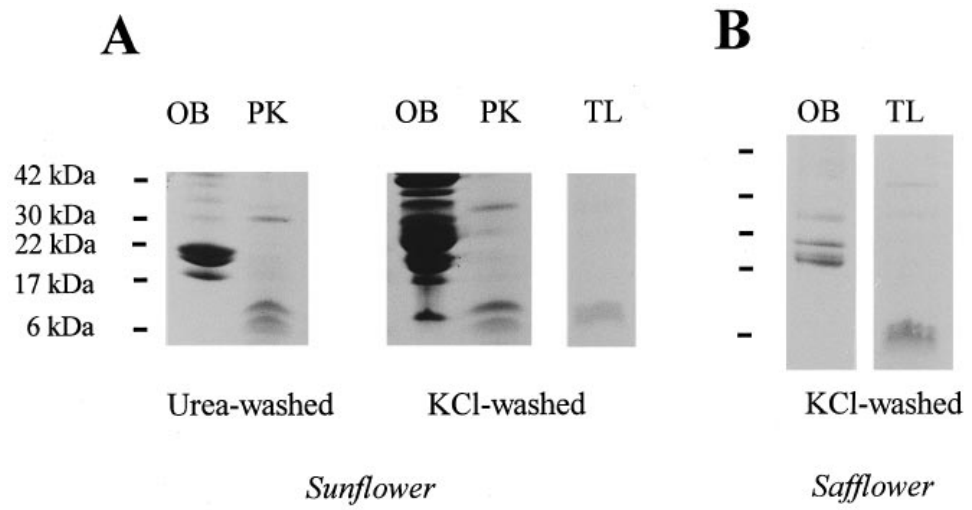

Figure 2 Peptide composition of oil body preparations from sunflower (A) and safflower (B) after proteolysis

Protein and peptide composition of the intact oil bodies (OB) and after protease digestion with either proteinase K (PK) or thermolysin (TL). The positions of molecular-mass markers are shown on the left.

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citrus     . . . . . . . . . .  . . . . . . . . . .  . . . . . . M A E H  Y . . Q P L Q . Q T  Q . . . . . L Q S R  Q P R S H Q V V K A  26
almond     . . . . . . . . . .  . . . . . . . . . .  . . . . . . M A D Q  H F Q Q P L H F Q G  S . . . . . Y G Q Q  Q P R S Y Q V A K A  29
canola     M T D T A R T H H D  I T S R D Q Y P R D  R D Q Y S M I G R D  R D Q Y S M M G R D  R D Q Y N M Y G R D  Y S K S R Q I A K A  60
sunflower  . . . . . . . . . .  . . . . M A T T T Y  D R H H V T T T Q P  Q Y R H D Q H T G D  R L T H P Q R H E Q  G P S T G K I M V I  46

citrus     A T A V T A G G S L  L V L S G L T M A G  T V I A L T I A T P  L L V I C S P V L V  P A V I T V S L L I  M G F L A S G G F G  86
almond     A T A V T A G G S L  L V L S G L V L A G  T V I A L T I A T P  L L V I F S P V L V  P A L I T V A L I T  M G F L T S G G F G  89
canola     V T A V T A G G S L  L V L S S L T L V G  T V I A L T V A T P  L L V I F S P I L V  P A L I T V A M L I  T G F L S S G G F G  120
sunflower  M A L L P I T G I L  F G L A G I T L V G  T V I G L P L A T P  L F V I F S P V I V  P A M I A I G L A V  T G F L T S G T F G  106

citrus     V A A I S V L S W I  Y R Y V T G G H P P  G A D Q L E Q A R M  K L . . . . . A  G K A R E M R D R A  E Q F G Q Q Q I T G  139
almond     V A A V T V L S W I  Y K Y V T G K Q P P  G A D Q L D Q A R H  K L . . . . . A  G K A R D I K D R A  E Q F G Q Q H V P S  142
canola     I A A I T V F S W I  Y K Y A T G E H P Q  G S D K L D S A R M  K L . . . . . G  S K A Q D L K D R A  Q Y Y G Q Q H T G G  173
sunflower  L T G L S S L S Y L  F N M V R R S T M S  V P D Q M D Y V K G  K L Q D V G E Y T G  Q K T K D L G Q K I  Q H T A H E M G D Q  166

citrus     S Q Q G S . . . . .  . . . . . .  . . 144
almond     G Q Q Q S S . . . .  . . . . . .  . . 148
canola     Y G Q Q H T G G E H  D R D R T R G T Q H  T T 195
sunflower  G Q G Q G Q G G G K  E G R K E G G K . .  . . 184
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Figure 3 Alignment of deduced amino acid sequences of oleosins from dicotyledonous plants

The N-terminal sequence of the 8 kDa proteinase-protected fragment from safflower (Leu-Ala-Gly-Gly-Ser) aligns with conserved oleosin sequences at the position indicated by the bar.

hydrophobic domain of the oleosins, buried within the core of the oil bodies, was provided by Western blots using a polyclonal antiserum raised against intact sunflower oil bodies. This antiserum reacted with the intact oleosins present in the safflower and sunflower oil bodies, but not with the protected fragments (results not shown). The characteristics of the protected fragments and the results of both the Western blotting and N-terminal sequencing experiments are in agreement with the models of Tzen et al. [14] and Li et al. [11], in which the hydrophobic domain of the oleosins is proposed to be buried in the TAG core of the oil body.

The secondary structures of the oleosins in salt- and/or urea-washed oil bodies and in oil bodies containing the protected fragments were analysed by FTIR spectroscopy. The TAGs that comprise some 95 % of the mass of oil bodies were responsible for most of the characteristics of the spectrum obtained from intact oil bodies (Figures 4A and 4B). Their most prominent bands arise from C–H (3050–2800 cm^{-1}) and C = O (1738 cm^{-1}) stretch vibrations, C–H deformation (1454 cm^{-1}) and C–O (1200–1100 cm^{-1}) bonding. These very strong bands could not be subtracted completely without distortions, as the remainder of the band at 1738 cm^{-1} shows. However, the amide bands of the proteins (the amide A at around 3295 cm^{-1}, amide I at 1656 cm^{-1} and amide II at 1546 cm^{-1}) were clearly visible in regions where pure oil did not have any strong absorptions. The small oil band at 1650 cm^{-1} could be compensated for by spectral subtraction. The positions of the amide I band maximum at 1656 cm^{-1} and the amide II band maximum at 1546 cm^{-1} in the spectrum of oleosins in salt-washed safflower oil bodies are very characteristic for α-helical structures (Figure 4C) and calculation as a percentage of the areas of amide I bands indicated that α-helices accounted for 50 % of the secondary structure of the dry protein (Table 2A). Shoulders at 1641 and 1628 cm^{-1} indicated parallel and antiparallel β-sheet structures, the latter possibly intermolecular. However, there appeared to be only a small amount of turn structures (1671 cm^{-1}) ($\cong$ 6 %, see Table 2A).

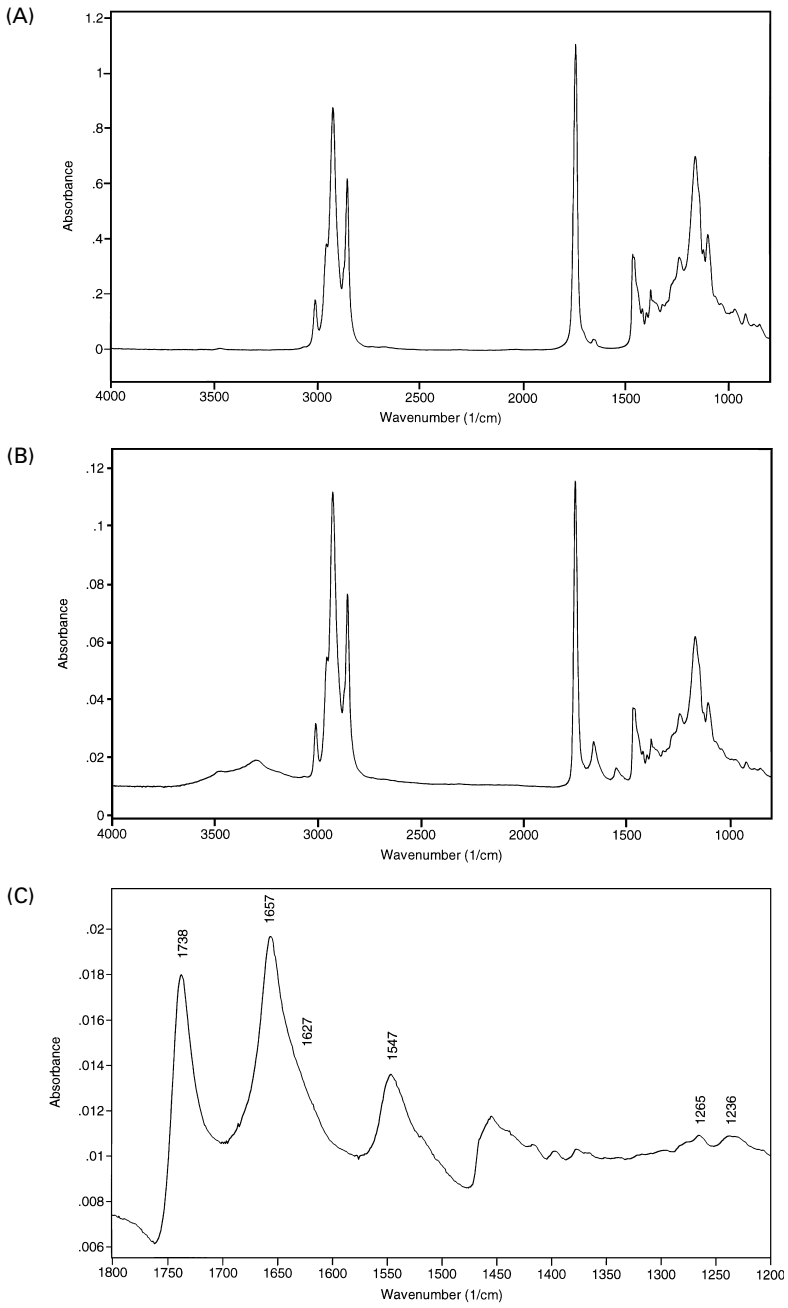

Figure 4 FTIR spectra of (A) pure safflower oil, (B) salt-washed safflower oil bodies and (C) the amide region of salt-washed safflower oil bodies

B and **C** were determined on air-dried films, after compensation for the oil as far as possible.

The spectrum of the oleosins in safflower oil bodies after urea treatment (Figure 5A) was very similar to that obtained for the salt-washed preparation (Figure 4C), with identical amide I and amide II band maxima but a slightly more pronounced shoulder at 1626 cm^{-1}. Lower intensities of the 1656 and 1640 cm^{-1} bands showed that the contents of α-helix and regular β-sheet were both slightly lower, whereas the bands at 1626 and 1682 cm^{-1} were increased (Table 2A). This suggests that denaturation and intermolecular aggregation of the oleosins may occur during or after urea treatment. However, the α-helical part of the protein is either largely protected from denaturation by urea or refolds on removal of the denaturant. It is unfortunate that the protein structure could not be determined in the presence of urea, because of the strong overlaps between the bands of urea and the amide bands of the protein.

After partial digestion of salt-washed safflower oil bodies with proteinase, the amide I band at 1657 cm^{-1} appeared narrower (Figure 5B). Comparison of the amide I bands of the two samples (Figure 5C) revealed that the shoulder due to β-sheet was noticeably smaller and there was a slight loss of absorption intensity in the 1690–1665 cm^{-1} region. Further analysis of this spectrum shows that the fragments remaining after proteinase digestion comprised 60 % α-helix, whereas the amounts of β-sheet and turn structures were reduced accordingly (Table 2A).

Table 2 Estimates of secondary structure contents of oleosins in air-dried films of safflower and sunflower oilbodies, based on percentage areas of amide I band components (cm^{-1})

Results are calculated from the spectra shown in Figures 4(C) and 5(A,B) and 6. Conformational assignments are as in Table 1.

A. Safflower

	Salt-washed		Urea-washed		Proteinase-treated	
Conformation	Band	Area (%)	Band	Area (%)	Band	Area (%)
β-sheet/β-turns	1690	1	1696	1	1690	1
β-sheet	1682	3	1682	12	1680	4
β-turns	1671	6	1670	8	1670	5
α-helix	1657	50	1656	46	1656	60
β-sheet	1641	26	1640	20	1641	20
	1628	5	1626	9	1629	5
	1618	9	1614	2	1618	6

B. Sunflower

	Salt-washed		Urea-washed		Proteinase-treated		Urea-washed + proteinase-treated	
Conformation	Band	Area (%)	Band	Area (%)	Band	Area (%)	Band	Area (%)
β-sheet/β-turns	1687	10	1696	2				
β-sheet	1678	2	1681	13	1681	2	1678	1
β-turns	1668	23	1667	15	1667	11	1668	8
α-helix	1654	33	1654	41	1655	59	1655	52
β-sheet	1639	22	1640	14	1640	8	1640	8
	1628	4	1628	12	1632	13	1628	21
	1621	6	1614	3	1617	7	1611	4
							1591	5

The salt-washed sunflower oil bodies contained many proteins in addition to oleosins (Figure 1), and the FTIR spectrum showed a mixture of secondary structures (Figure 6). The peaks of the amide I band at 1654 cm^{-1} and the amide II band at 1547 cm^{-1} showed a considerable amount of α-helix, accounting for about 30 % of the protein secondary structures, but some unordered structures may also be present. Prominent shoulders at 1639 cm^{-1} and at 1520 cm^{-1} indicated about 35 % β-sheet, whereas the strong absorption in the 1700–1660 cm^{-1} region and around 1560 cm^{-1} showed the presence of about 25 % turn structure (Table 2B).

Urea treatment of the sunflower oil bodies resulted in removal of non-oleosin proteins (Figure 1). The amounts of β-sheet and turn structures (Figure 6) also decreased, whereas the amount of α-helix increased to 41 % (Table 2B). The FTIR spectrum of the proteinase-treated oil bodies (Figure 6) showed a clearly α-helical structure, resembling that of the protected fragments of safflower oleosins (Figure 5B). Both samples contained about 60 % of α-helix, but there were small differences in the amounts of β-sheet and turns (Table 2B). When proteinase treatment was performed on urea-washed sunflower oil bodies (Figure 6), the protected fragment retained most of its α-helical content, which fell to 52 % from 59 % (Table 2B). However, an increase in the shoulder at 1628 cm^{-1} showed intermolecular β-sheet resulting from aggregation, suggesting that urea treatment may have some effect on the hydrophobic domain of sunflower oleosins.

The spectra shown in Figures 4–6 and the structure determinations based on them were obtained with films of oil bodies deposited on the ZnSe ATR crystal and dried in a stream of dry air (see the Materials and methods section). This gave the best spectral quality, but raises the possibility that oleosins or oleosin fragments may have become partially denatured or changed their conformations from that in oil bodies suspended in aqueous medium. To rule out this possibility, FTIR spectra were also determined on salt-washed and proteinase-treated oil bodies of sunflower (Figure 7) and safflower (results not shown) while suspended in aqueous medium.

The spectrum of the salt-washed oil bodies of sunflower (Figure 7A) in water was similar to that determined for the same preparations in the dry state (Figures 6 and 7B), but the shoulders due to β-sheet and turn structures were much more pronounced, which makes the amide I band appear broader. This difference may in part be an effect of the protein hydration, which causes shifts in the main band positions of β-sheet and turn structures (1640–1637 and 1667–1672). As a consequence of the changing band-overlaps in the amide I region, the α-helix content may be overestimated in the dry state and underestimated in the wet samples. However, a genuine structure change may also occur when proteins are transferred from an aqueous into an anhydrous lipid environment, which would favour the formation of α-helices [28]. If this were to happen during the drying, the structures outside the lipid phase would be affected, while the buried parts of the protein would be unchanged. The large error margins of structure determinations carried out in the aqueous systems cannot rule out such effects completely, but the analyses of the aqueous samples indicated that the proteinase-treated fragments of sunflower and safflower oil bodies contained similar amounts of α-helical structure, about 40–45 %, giving a lower limit for the estimate of this structure. The validity of the data is also shown by the identical effects of proteinase treatment on the aqueous and dried sunflower oil body samples (Figures 7A and 7B), both showing increased amounts of α-helical structure at the expense of β-sheet and turns.

In conclusion, the FTIR spectroscopy analyses of the oil bodies indicated that sunflower oleosins may be more susceptible to denaturation by urea treatment than those of safflower oil bodies, and that care must be taken in using this strategy to purify oil bodies from sunflower and other species which may

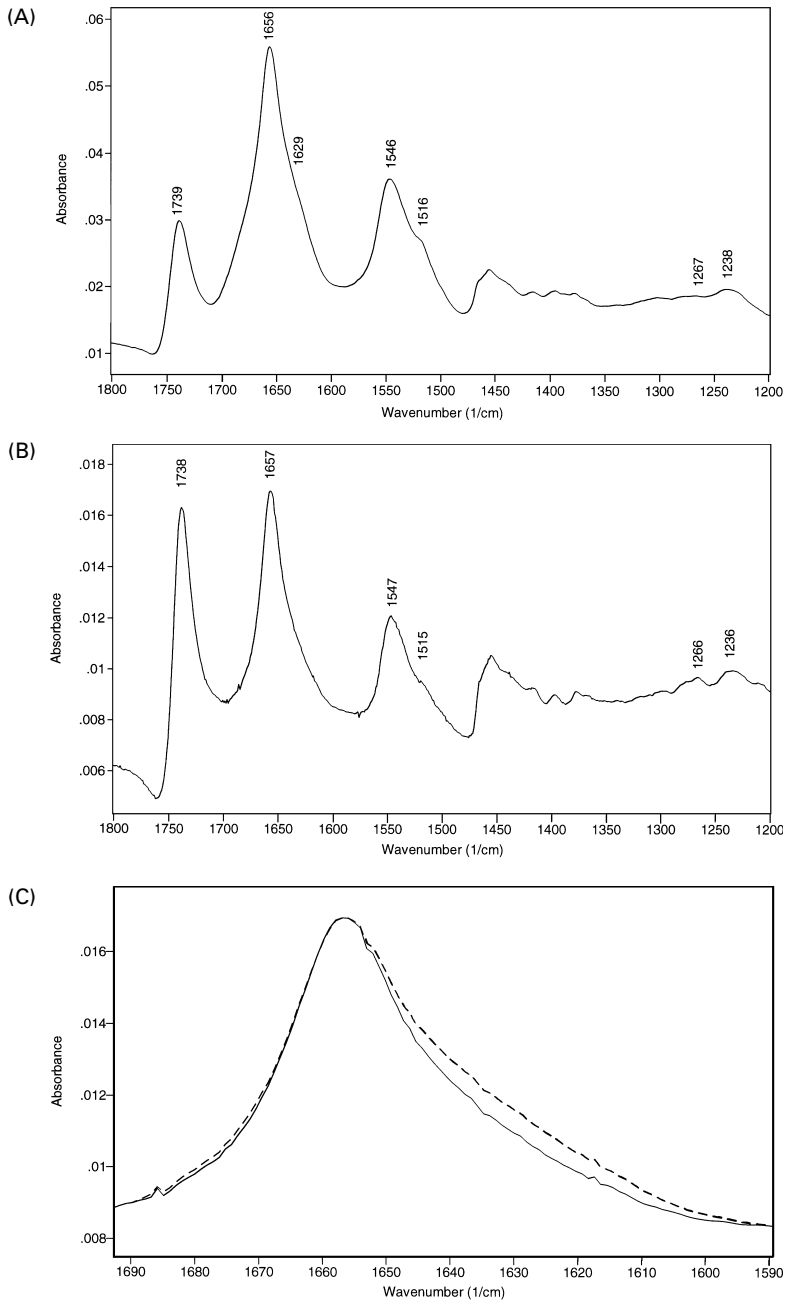

Figure 5 FTIR spectra of (A) urea-washed safflower oil bodies, (B) proteinase-treated safflower oil bodies and (C) amide I bands of salt-washed safflower oil bodies (broken line) and proteinase-treated safflower oil bodies (solid line)

All spectra are determined on air-dried films and compensated for oil as far as possible.

show similar properties. However, it is clear that the hydrophobic domains of the sunflower and safflower oleosins have similar α-helical secondary structures.

DISCUSSION

In order to determine the secondary structure of oleosins, it was initially necessary to evaluate methods for preparing safflower and sunflower oil bodies containing oleosins but no other contaminating proteins (Figure 1). Such oil bodies were purified from safflower by salt-washing, whereas similar oil bodies from sunflower could only be prepared by urea-washing. The FTIR spectroscopy showed that treatment of safflower oil bodies with urea had little effect on the secondary structure content of the protein (Figures 4 and 5), although similar treatment may have affected the sunflower preparation (Figure 6). Proteinase treatment of salt-washed oil bodies isolated from both species showed that two doublets of about 8 and 6 kDa were protected from digestion and were therefore likely to be buried within the oil body (Figure 2). That the 8 kDa fragment of safflower was derived from the central hydrophobic domain of the oleosins was demonstrated by N-terminal sequencing. The origin of the 6 kDa

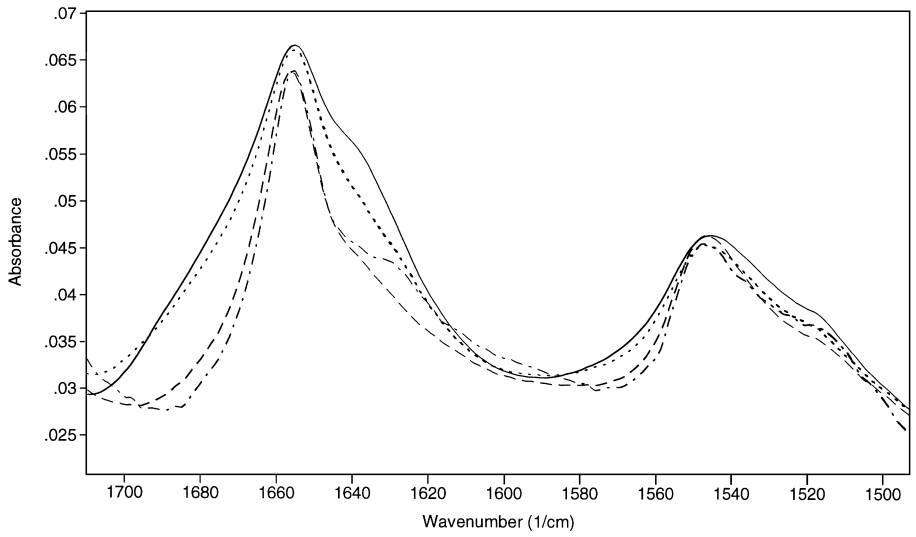

Figure 6 FTIR spectra of salt-washed sunflower oil bodies (solid line), urea-washed sunflower oil bodies (dotted line), proteinase-treated sunflower oil bodies (broken line) and urea-washed and proteinase-treated sunflower oil bodies (dotted/broken line)

All spectra are determined on air-dried films and compensated for oil as far as possible.

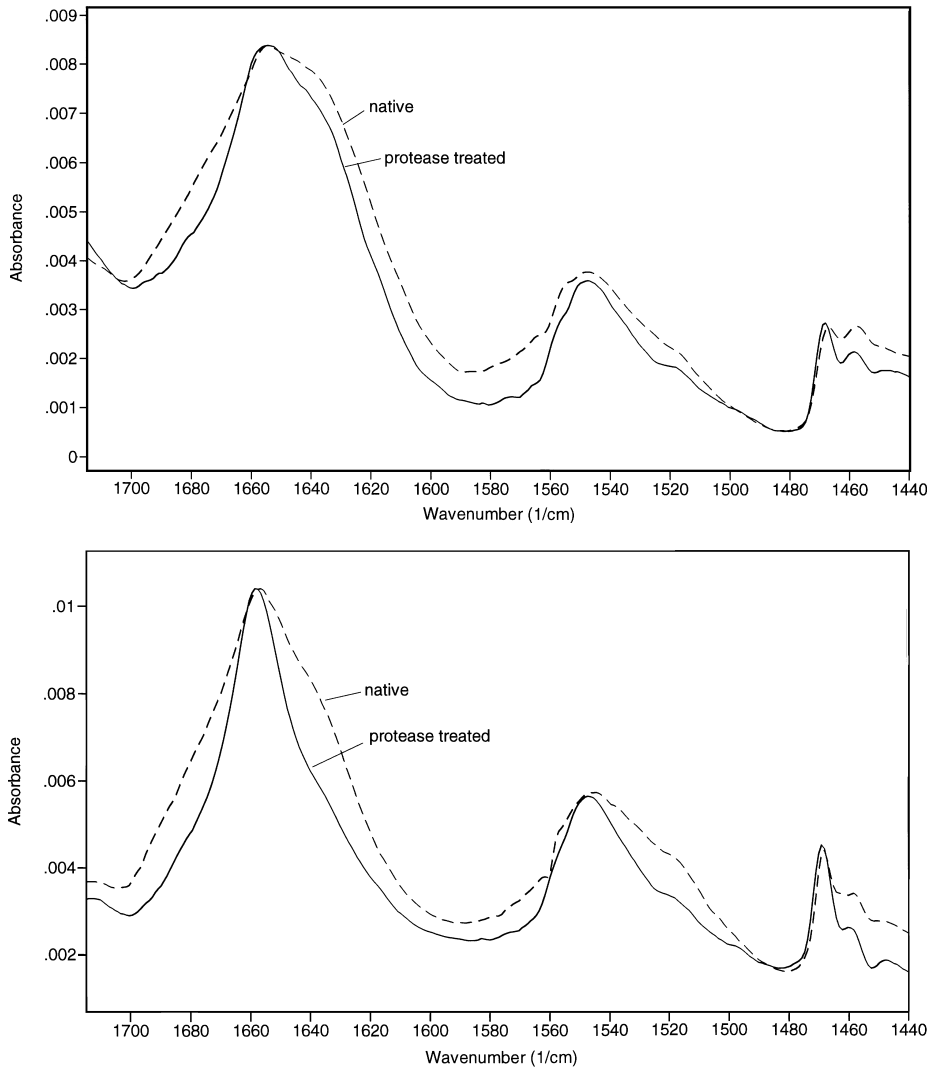

Figure 7 FTIR spectra of (top panel) salt-washed and proteinase-protected oil bodies of sunflower in aqueous medium, (bottom panel) the same samples as the top panel but in the dry state

fragment was not determined, but it appeared to result from further digestion of the 8 kDa fragment. The protected oleosin fragments from both species contained almost only α-helical structure (Figures 5B and 6). Similarly, the salt- and urea-washed oil bodies of both species were rich in α-helix but also contained some β-sheet structure, the amount of which varied with the sample (Figures 4, 5A and 6). The results are consistent with the CD analyses of purified sunflower oleosins described by Millichip et al. [17], although that study was carried out on proteins removed from the unique environment of the oil body.

Our results suggest a revised model for the secondary structure of oleosins. Previous studies have suggested that the C-terminal domain of the oleosin molecule may contain amphipathic α-helical structure [1,11,14] and our results are consistent with this. The N-terminal domain is more variable in sequence and has been suggested to contain a significant amount of β-strand structure [16]. We have demonstrated the presence of β-strand structure in the intact oleosin molecules, which then disappears upon protease digestion, consistent with the presence of this structure in either the N-terminal or C-terminal regions. It is possible, therefore, that the β-strand structure is present in the N-terminus of the oleosin molecule, as suggested previously [16], and may act as a lipase-docking site during seed germination, as hypothesized by Li et al. [16]. The central hydrophobic domain that binds the oleosin molecule into the TAG core is remodelled significantly from the current structural model. Instead of having a β-sheet structure, anti-parallel or otherwise, it appears that this domain of the oleosin is essentially α-helical in structure. This may not be surprising when it is considered that the hydrophobic domains of membrane proteins are usually α-helical in structure. It is therefore likely that the hydrophobic domain is comprised of two α-helices, with the 'proline knot' forming an 180° turn. This model is in agreement with the initial proposal of the secondary structure of the oleosin molecule made by Vance and Huang [12].

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