BIOCHEMICAL JOURNAL LETTERS

Oil-bodies from sunflower (*Helianthus annuus* L.) seeds

Storage triacylglycerols (TAG) in plant seeds are present in small discrete intracellular organelles ranging from 1 to 2 μm, which are called oil-bodies. Oil-bodies are abundant in plant seeds and are among the simplest organelles present in eukaryotes. They are remarkably stable both inside the cells and in isolated preparations. In both situations the organelles occur as individual entities, and when they are pressed against one another *in vivo* during seed desiccation or *in vitro* after flotation centrifugation, they do not aggregate or coalesce, even after prolonged storage [1].

In 1992, Tzen and Huang [2] determined the chemical composition of maize (*Zea mays*) oil-bodies and proposed a particular structure possibly explaining their great stability. According to these authors, an oil-body has a matrix of TAG which is surrounded by a monolayer of phospholipids (PL) and alkaline proteins named oleosins. Oleosins, shield the PL shell so that the PL are not accessible to external phopholipase A_2 and phospholipase C. At present, this structure is thought to be common to all oil-bodies, although no such precise measurements as those carried out by Tzen and Huang [2] have been made on other material.

In a recent paper, Millichip et al. [3] contested the general validity of the Tzen and Huang's [2] model, claiming that, in sunflower (*Helianthus annuus* L.) oil-bodies, the phospholipid content was less than 0.004 % of that required to form a half-unit membrane surrounding the oil-body. They therefore concluded that, in sunflower, the oil-body surface appears to have an unusual structure and to be largely covered by an oleosin protein coat rather than a half-unit membrane. These conclusions are at variance with the results we have obtained at our laboratory with oil-bodies originating from the some plant species. We therefore examined the results of Millichip et al. [3] in detail and observe that their conclusions are far from being reliable, as we have detected errors in their calculations.

According to Millichip et al. [3], the PL content of a urea-purified oil-body with a diameter of 1 μm is 3.46×10^{-19} g (see [3], p. 336) and the percentage (w/w) of PL in the total lipids, 0.7 % $\pm$ 0.1 %. The last value corresponds to a PL/TAG ratio of 0.71 % [3]. On the basis of this value we calculated the TAG content of one oil-body, which works out at 3.46×10^{-19} g/0.0071 $= 4.87 \times 10^{-17}$ g. Taking the TAG mass per unit volume to be 0.92 g/cm^3 [2], the volume occupied by the TAG content of one oil-body is $4.87 \times 10^{-17}/0.92 = 5.29 \times 10^4$ nm^3. As the volume of a 1 μm oil-body is $(4/3) \times \pi \times (500)^3 = 5.24 \times 10^8$ nm^3, this means that the volume occupied by TAG would be about a ten-thousandth of the oil-body volume. It therefore seems likely that one of the two values given by Millichip et al. [3], 3.46×10^{-19} g of PL and 0.7 % of PL in lipids, may be wrong. As regards the second value, it should be noted that it is in the same range as that reported by Tzen and Huang for maize [2] and as that obtained at our laboratory for sunflower oil-bodies prepared from dry seeds (Rustica, Euroflor variety) as described by Tzen and Huang [2] (see Table 1).

Table 1 Ratio of PL to TAG in oil-bodies of various species as found in various studies

Species	Ratio PL/TAG (%)	Reference
Maize	0.93	[2]
Sunflower	0.71	[3]
	1.3 $\pm$ 0.3	The present study

These differences are small and may reflect variations in the size of oil-bodies originating from different species or cultivars [4]. We are therefore convinced that it is the value proposed for the PL content which is wrong. The PL/TAG ratio is probably correct, and we can show that this value is close to the theoretical ratio needed to cover the oil-body surface with a PL monolayer.

Taking an average oil-body diameter of 1.5 μm, it is possible to calculate the mean oil-body volume, which works out at $(4/3) \times \pi \times (750)^3$ nm^3 $= 1.76 \times 10^9$ nm^3. As the thickness of a PL monolayer is 2.5 nm [2], the volume of TAG present in one oil-body is $(4/3) \times \pi \times (750 - 2.5)^3$ nm^3 $= 1.749 \times 10^9$ nm^3 and the volume of a theoretical continuous PL monolayer is $(1.767 \times 10^9 - 1.749 \times 10^9)$ nm^3 $= 0.18 \times 10^8$ nm^3.

Taking 0.92 g/cm^3 $= 0.92 \times 10^{-2}$ g/nm^3 and 1.03 g/cm^3 $= 1.03 \times 10^{-21}$ g/nm^3 as the mass per unit volume for TAG and PL respectively [2], the PL/TAG ratio compatible with total coverage of the oil-body surface by PL can be calculated. We obtained:

- TAG mass $= 0.92 \times 10^{-21} \times 1.749 \times 10^9$
  $= 1.609 \times 10^{-12}$ g/oil-body
- PL mass $= 1.03 \times 10^{-21} \times 0.18 \times 10^8$
  $= 0.185 \times 10^{-13}$ g/oil-body
- PL/TAG $= 0.185 \times 10^{-13}$ g/1.609 $\times 10^{-12}$ g
  $= 0.0115$, that is, 1.15 %

The simple calculation made above clearly shows that the PL/TAG ratio of sunflower oil-bodies obtained by Millichip et al. [3] is enough for a half-unit membrane covering about $(0.71/1.15) \times 100 = 62$ % of a 1.5 μm oil-body. The claim by Millichip et al. [3] that their results give PL values of less than 0.01 % of that required to cover the surface area of a sunflower oil-body [3] is not valid, probably because they made a mistake in calculating the PL content which can be said to be at least 6200 times too small (62/0.01). The calculation presented here does not prove the validity of Tzen and Huang's [2] model for all oil-bodies whatever their origin, but it seems the only one available

so far which is compatible with all the data obtained, including those on sunflower.

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1 Huang, A. H. C. (1985) in Modern Methods of Plant Analysis, vol. 1. (Linskins, H. F. and Jackson, J. F., eds.), pp. 145–151. Springer Verlag, Berlin
2 Tzen, J. T. C. and Huang, A. H. C. (1992) J. Cell Biol. **117**, 327–335
3 Millichip, M., Tatham, A. S., Jackson, F., Griffiths, G., Shewry, P. R. and Stobart, K. (1996) Biochem. J. **314**, 333–337
4 Tzen, J. T. C., Cao, Y. Z., Laurent, P., Ratnayake, C. and Huang, A. H. C. (1993) Plant Physiol. **101**, 267–276

Received 26 March 1996

Oleosins and oil bodies in plant seeds have postulated structures

In plant seeds, storage oils [triacylglycerols (TAGs)] are present in subcellular spherical oil bodies of about 0.6–2 μm in diameter [1]. Each oil body has a matrix of TAGs surrounded by a layer of phospholipids (PL) embedded with abundant structural proteins called oleosins. Oleosins have M_r values between 15000 and 26000, depending on the isoforms and plant species in which they occur. They completely cover the surface of the oil bodies and prevent the PL layers of adjacent oil bodies from contacting and the oil bodies from coalescing. Maintaining the oil bodies as small individual entities provides a large surface area per unit TAG for lipase to act on during seed germination.

Oleosins have unique secondary structures which interact with other molecules on the surface of the organelles. On the basis of considerations of thermodynamics and experimental findings [1–3], these secondary structures have been postulated to be (a) an N-terminal amphipathic stretch (20–60 residues) of an undefined structure residing on the organelle surface, (b) a central hydrophobic domain (72 residues) of long antiparallel β-structures penetrating into the matrix and (c) a C-terminal amphipathic α-helix (30–40 residues) positioning on the organelle surface. Several laboratories have arrived at similar postulations or findings, even though there are minor disagreements.

Recently, Millichip and co-workers [4] reported that oil bodies isolated from maturing sunflower (*Helianthus annuus* L.) seeds were heavily contaminated and that washing the oil-body fraction repeatedly with 9 M urea removed the contaminants but did not affect the organelle size [4]. Oleosins extracted from these urea-washed oil bodies did not exhibit the postulated β-structures when examined by IR and UV CD spectrometry. The washed oil-body fraction contained an amount of PL that was judged to be grossly insufficient to form one layer covering the surface of the oil bodies, and it had a minimal amount of acidic lipids. The authors concluded that the oil bodies and oleosins did not have the postulated structures described above. They extended their findings and interpretations to the oil bodies and oleosins in mature seeds and other plant species.

The above findings [4] might have been due in part to the harsh procedure (with 9 M urea) used to isolate the oil bodies. Whether these isolated organelles are similar to those *in vivo* in structure and properties needs to be assessed. The authors of the recent report [4] only assessed the successful maintenance of the organelle size with the use of an optical microscope. We performed a similar experiment and confirmed by optical microscopy that there was no major difference in size between the control and the urea-washed oil bodies isolated from mature sunflower and maize seeds. However, it was difficult to judge whether there were actual differences in size (e.g. twice or one half), and especially whether there were granular materials on the surface [4]. Regardless, the mere observation that the urea-washed oil bodies remained as individual entities is not a reliable criterion that the organelles, especially the component on the surface, had not been altered by the strong chaotic reagent. In addition, it is uncertain whether all of the materials washed away by the 9 M urea were contaminants [4] or authentic oil-body components.

The effects of urea and other strong reagents on the oleosin structures should be evaluated. In the recent report [4], the oil bodies were washed repeatedly with 9 M urea; the urea would denature the oleosins on the organelle surface regardless of whether the size of the organelles had been preserved. Specifically, urea would disrupt, to a large or small extent, the hydrophilic and hydrophobic interactions within the protein, between individual proteins, and between the protein and the underlining amphipathic and neutral lipids. Then the oil bodies were treated with acetone and diethyl ether to remove the associated lipids. The remaining oleosins were dissolved in trifluoroacetic acid, a strong acid with a pK of 0.25, before they were separated by HPLC. The separated oleosins were re-dissolved in solvents, in the presence of SDS, for the structural analyses by IR and UV CD spectrometry. In view of the above harsh treatment and destruction of the unique environment of the oil-body surface, one would hardly expect the oleosins to be able to maintain their native structures, even in the presence of SDS micelles. Nevertheless, two laboratories used much milder conditions (without urea and trifluoroacetic acid) to isolate the oleosins and detected a high proportion of β-structures in the proteins [3,5]. These findings are in accord with the β-structures of the central hydrophobic domain postulated independently on the basis of thermodynamic considerations [1].

The question of whether there were sufficient PL forming one molecular layer covering the oil body was raised in the recent report [4]. It is unknown if washing the oil bodies with 9 M urea removed some PL authentic to the organelles. Regardless, using a published method of data analyses [6], the authors of the report [4] concluded that the amount of PL in the urea-washed oil-body fraction was less than 0.1 % of that required to form one PL layer surrounding an organelle of 1–2 μm (not accurately measured) in diameter. This conclusion is invalid because the report had a simple calculation error of a 10^4 magnitude. The urea-washed oil bodies of 1–2 μm in diameter contained 0.7 % (w/w) PL and 98 % TAGs [4]. Calculated from these values, an oil-body of 1 and 2 μm in diameter had 3.4×10^{-15} (instead of 3.4×10^{-19} [4]) and 2.7×10^{-14} (instead of 2.7×10^{-18}) g of PL respectively. The amounts of PL (recalculated to be 2.7×10^{-14} g) are actually sufficient to form one layer of PL on the surface of an oil body of 2 μm in diameter [6]. Variations in the percentage volume of the PL shell occupied by oleosins and the standard errors in quantitative analysis of the TAGs and PL will not alter the outcome substantially. Earlier, different laboratories reported that oil-body fractions isolated from the seeds of diverse plant species contained substantial amounts of PL [1]. Some of these PL could be contaminants of the oil-body fractions. Nevertheless, the recent report [4] specifically cited one paper showing that the oil-body fraction isolated from carrot (*Daucus carota*) cultures had little PL [7]. However, this carrot oil-body fraction had abundant non-esterified fatty acids (either authentic or derived from PL and TAGs), but possessed no unique proteins (oleosins), and the authors actually wondered whether the oil-bodies in the carrot cultures were different from those in seeds.

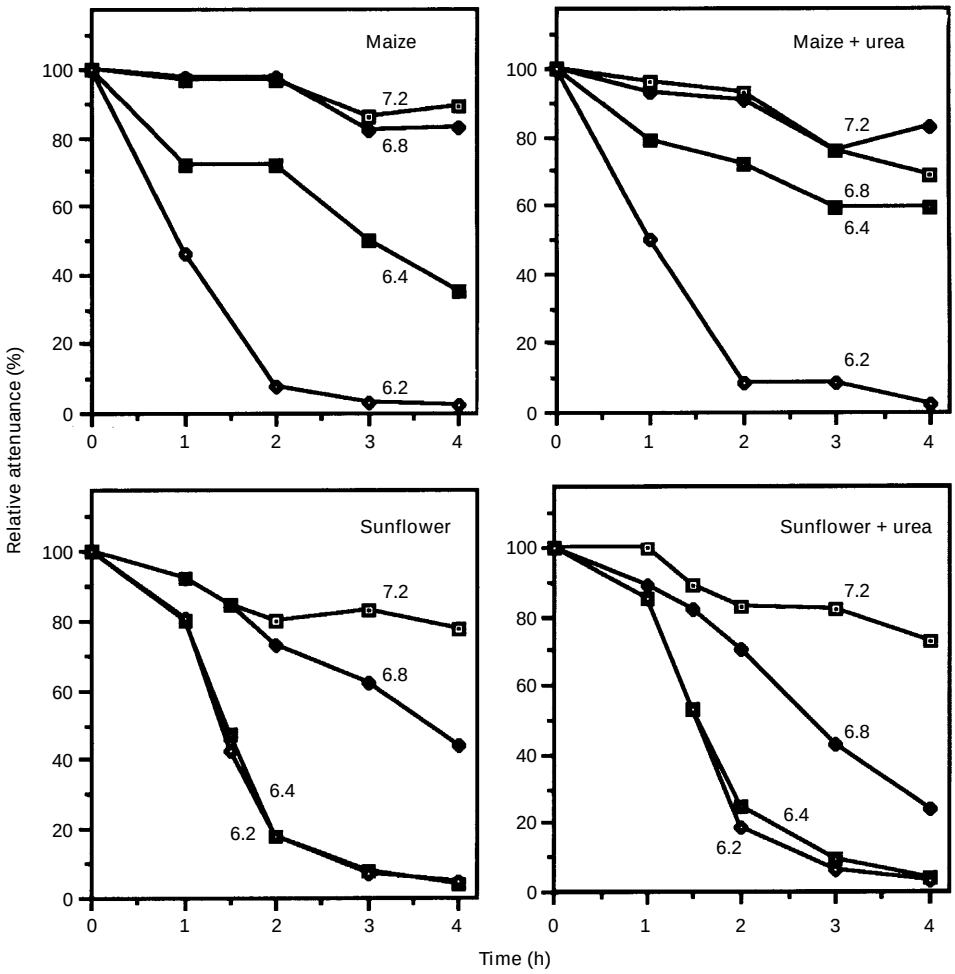

Figure 1 Aggregation of oil bodies in a suspension was induced by lowering the pH of the medium

Oil bodies were isolated from mature maize (*Zea mays*) and sunflower seeds and washed or not washed with 9 M urea. They were suspended in 0.25 M sucrose and 50 mM Bistris at the pH indicated. Each 1.4 ml suspension was placed in a 1.8 ml cuvette, and the relative attenuance ($10^{D}/10^{D_0}$) at 600 nm of the lower portion of the suspension (approx. 0.5 ml) was measured at the indicated time intervals [2]. Aggregated oil bodies floated to the top of the suspension, and the attenuance of the suspension through which the light-path went was reduced [2].

Oil-body fractions isolated from the seeds of diverse plant species have been found to contain acidic PL and non-esterified fatty acids [1,2]. An analysis of the positions of the amino acid residues in the amphipathic stretches of the oleosin has led to the postulation that the oleosin molecule on the oil body has its basic residues facing internally to interact with the acidic lipids, whereas its acid residues are exposed to the exterior [2]. The recent report [4] found insufficient amounts of acidic lipids in the sunflower oil-body fraction and suggested that the oleosin somehow interacted with the matrix TAGs. Clearly, abundant acidic components must be present in the oil bodies to account for the following two findings reported by several laboratories. First, oleosins are basic proteins, as judged from computations of all the charges associated with the residues in oleosins of known amino acid sequences [1,2]. Oleosins are not known to be covalently linked to acidic carbohydrates or other moieties that could have altered the basic nature of the proteins, and they migrate as basic molecules during isoelectric focusing [8]. Secondly, the whole oil body has a pI of about 6, as shown by the reversible, pH-dependent aggregation of the organelles at this pH [2,9] and by isoelectric focusing of the organelles [2]. Because oleosins are abundant, covering the entire surface of the oil bodies [6], there must be substantial amounts of acid components to counter the basic residues of the oleosins, such that the organelle has an overall pI of 6. In short, information obtained from different laboratories suggests that there are abundant acidic components on the oil bodies. The possibility could exist that the acidic components were contaminants attached to the oil bodies and were removed by urea washing. This was not the case, because we observed that oil bodies, washed or not washed with urea, still exhibited a pI of about 6 in an analysis of the pH-dependent aggregation of the organelles (Figure 1). Acidic components, lipids or otherwise, must be associated with the oil bodies on the surface.

The idea of an oil body being surrounded by a layer of PL was first proposed on the basis of the hydropathic properties of the molecules involved and by electron-microscopic observations [10]. Certainly, the presence of an electron-dense line, even if it could be measured to a 2–3 nm thickness, around oil bodies fixed with osmium does not definitively prove the presence of a half-unit membrane. Conversely, the absence of such an electron-dense line does not preclude the existence of a half-unit membrane because of the variations in the electron-microscopic procedure. The recent report [4] cited an earlier study that failed to find a

membrane, half-unit or otherwise, on the surface of oil bodies in castor bean (*Ricinus communis*) [11]. A closer examination of this study and another paper [7] mentioned in the report reveals the contrary. The electron micrographs in these two papers show that, after osmium fixation, many, although not all, of the oil bodies *in situ* were surrounded by an electron-dense line.

In view of the earlier voluminous findings and the lack of convincing evidence and arguments from the recent report [4], we maintain that the oil bodies and oleosins in plant seeds have the postulated structures.

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Received 1 April 1996

1 Huang A. H. C. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. **43**, 177–200
2 Tzen, J. T. C., Lie, G. C. and Huang, A. H. C. (1992) J. Biol. Chem. **267**, 15626–15834
3 Li, M., Smith, L. J., Clark, D. C., Wilson, R. and Murphy, D. J. (1992) J. Biol. Chem. **267**, 8245–8253
4 Millichip, M., Tatham, A., Jackson, F., Griffiths, G., Shewry, P. R. and Stobart, K. (1996) Biochem. J. **314**, 333–337
5 Jacks, T. J., Hensarling, T. P., Neucere, J. N., Yatsu, L. Y. and Barker, R. H. (1990) J. Am. Oil Chem. Soc. **67**, 353–361
6 Tzen, J. T. C. and Huang, A. H. C. (1992) J. Cell Biol. **117**, 327–335
7 Kleinig, H., Steinke, C., Kopp, C. and Zarr, K. (1978) Planta **140**, 233–237
8 Qu, R., Wang, S. M., Lin, Y. H., Vance, V. B. and Huang, A. H. C. (1986) Biochem. J. **234**, 57–65
9 Slack, C. R., Bertaud, W. S., Shaw, B. P., Holland, R., Browse, J. and Wright, H. (1980) Biochem. J. **190**, 551–561
10 Yatsu, L. Y. and Jacks, T. J. (1972) Plant Physiol. **49**, 937–943
11 Harwood, J. L., Sodja, A. and Stumpf, P. K. (1971) Lipids **6**, 851–854