Tocopherol—An Intrinsic Component of Sunflower Seed Oil Bodies

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ABSTRACT: Oil bodies were removed from mature sunflower through wet grinding followed by filtration then centrifugation and recovered as the buoyant fraction. Washing this fraction with buffer (water-washed oil bodies, WWOB) or 9 M urea (urea-washed oil bodies, UWOB) resulted in the removal of extraneous proteins. SDS-PAGE of the proteins still associated with the oil body fraction after washing indicated that this effect was particularly dramatic with urea washing. Thirty-eight percent of the total seed tocopherol was recovered in WWOB after only one cycle of oil body recovery. The total phenolic content (TPC) of differentially washed sunflower seed oil bodies was used as a marker for the nonspecific association of phenolic compounds to oil bodies. This value decreased with increased removal of proteins from oil bodies, whereas the converse was true for tocopherol values, which increased from 214 mg total tocopherol kg^{-1} WWOB [dry wt basis (dwb)] to 392 mg total tocopherol kg^{-1} UWOB (dwb). The ratio of the four tocopherol isomers remained constant in the seed and oil body preparations (α:β:γ:δ approximately 94:5:0.5:0.5). This work provides evidence that an intrinsic population of tocopherol molecules exists in the oil bodies of mature sunflower seeds.

Paper no. J11132 in *JAOCS 83*, 341–344 (April 2006).

KEY WORDS: Oil body, sunflower, tocopherol.

Oil bodies are small, discrete plant organelles found principally within the seeds of oilseeds; their primary function is to store neutral lipids during seed dormancy. Oil bodies of oilseeds such as sunflower (*Helianthus annuus* L.) range in size from 0.1 to 2.5 μm *in vivo* (1) and are formed of a central neutral lipid core (94–98% w/w) surrounded by a phospholipid monolayer (0.5–2% w/w) and a coat of strongly amphiphilic oleosin and caleosin proteins (0.5–3.5% w/w) (2).

Tocopherol consists of a group of lipophilic, monophenolic antioxidants. They have a wide range of biological functions within plant cells. In addition to acting as a membrane stabilizer and providing protection against oxygen toxicity (3), they are involved in intracellular signaling and cyclic electron transport around photosystem II (4). Although work by Sattler *et al.* (5) indicated that the necessary level of antioxidant protection within seeds would require tocopherol to be associated with oil bodies, no one has addressed fully the intracellular location of tocopherol in mature oilseeds.

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To date, the only reports on tocopherol distribution within oil seeds of which the authors are aware are a 1968 paper discussing the intracellular location of tocopherol in soybean, which concluded that tocopherol is not contained within the storage lipids (6), and a 1976 paper concerning the effect of soybean germination on crude tocopherol distribution (7), which observed the enrichment of γ-tocopherol in a spherosome fraction that floated to the top on centrifugation. This was a passing observation and not central to their interest in chloroplast development. In this paper we report on work to establish whether tocopherol is actually associated with oil bodies (spherosomes).

Oil body extraction techniques have been developed primarily for the biochemical and structural analysis of oleosin and caleosin, the major structural proteins that are an integral component of the organelle. Lacey *et al.* (8) extracted sunflower oil bodies and purified them by washing with 9 M urea. This chaotropic agent effectively removed extraneous proteins that were passively associated with oil bodies.

To establish whether tocopherol is an intrinsic component of sunflower oil bodies, the organelles were extracted and washed with urea to remove extraneous proteins and associated material; the tocopherol intrinsic to oil bodies was then measured.

EXPERIMENTAL PROCEDURES

All chemicals were standard laboratory reagent grade or higher and were sourced from Fisher Scientific (Loughborough, United Kingdom) unless otherwise stated. Precision protein stains and Coomassie blue R-250 were obtained from Bio-Rad (Hemel Hempstead, Herts, United Kingdom), and dehulled sunflower seeds were purchased from Lembas Ltd. (Sheffield, United Kingdom).

Oil body isolation. Oil bodies from dehulled sunflower seed were extracted and purified by the method of Tzen *et al.* (9) with slight modifications as described. Seeds (100 g) in grinding medium (0.5 L, 10 mM sodium phosphate pH 7.5, 0.6 M sucrose) were ground in a Kenwood blender (BL315 full power for 60 s). The slurry was filtered through three layers of cheesecloth and the filtrate centrifuged in 400-mL batches ($8000 \times g$ for 30 min at 5°C). The upper layer was isolated using a chilled metal spatula and dispersed (5 mL) in buffer (25 mL, 10 mM sodium phosphate, pH 7.5), then centrifuged at 5°C. (A swinging bucket rotor at $2000 \times g$ for 20 min was used for all other

centrifugations unless stated otherwise.) The upper layer was isolated and designated as a water-washed oil body preparation. This isolate was then purified by suspending the water-washed preparation (5 mL) in washing buffer (25 mL, 9 M urea, 10 mM sodium phosphate buffer, pH 7.5) and centrifuging at room temperature. The fat pad was isolated, resuspended (in 10 mM sodium phosphate buffer, pH 7.5), and centrifuged at room temperature to remove residual urea and extraneous proteins. After centrifugation the centrifuge tube was then chilled on ice for 5 min, and the upper solidified phase (urea-washed oil body preparation) was removed, mixed with sodium azide (0.02 M), and stored at 5°C under nitrogen.

Protein characterization. Isolated oil bodies (0.1 g) were suspended in distilled water (1 mL) and then vortexed (2 min); next, 100 µL of 100% TCA (wt/vol) was added. The tube was chilled on ice for 30 min to promote the precipitation of proteins and centrifuged (8500 × *g* for 5 min). The supernatant was aspirated and discarded and the pellet vortexed with 200 mL SDS solution (10% wt/vol SDS, 10 mM β-mercaptoethanol, 20% v/v glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% bromophenol blue.)

Proteins were resolved by SDS-PAGE using 15 and 4.0% polyacrylamide gels in the separating and stacking gel, respectively. After electrophoresis the gel was stained with Coomassie blue and destained with excess methanol/distilled H_2O/acetic acid (9:9:2 by vol).

Proximate analysis. The moisture content of oil body and seed samples was determined gravimetrically by drying to constant weight (vacuum oven, 60°C). Lipid content was calculated by repeated extraction with diethyl ether and determined gravimetrically (10). The protein content of the defatted residue was determined using the bicinchoninic acid assay (11) following solubilization of proteins in 2% SDS at 90°C using BSA as a standard. All analyses were carried out in triplicate.

Extraction and quantification of tocopherol. Sunflower seed was ground in a pestle and mortar after freezing in liquid nitrogen, then homogenized in a sample mill (2 × 2 s burst); the homogenized seed (5 g) or fresh oil-body cream (5 g) was then freeze-dried. Methanolic extraction was carried out with freeze-dried sample (1 g) in 100% methanol (8 mL) by using a roller mixer for 12 h; the supernatant was then aspirated after centrifugation (2000 RCF for 10 min). Methanolic extraction was repeated twice, and pooled samples were stored under nitrogen at –20°C.

Tocopherol content and composition were analyzed by HPLC as described by Bryngelsson *et al.* (12). Methanolic extract (1 mL) was evaporated to dryness in a rotary evaporator at 40°C and resuspended in hexane (1 mL), then syringe filtered (0.45 µm nylon filter).

HPLC was performed using a Waters 2695 separation module equipped with a Waters 996 photodiode array detector and a JASCO intelligent fluorescent detector P-920 (excitation set at 294 nm and emission at 326 nm, gain set at 10). Separations were performed using an Inertsil 5 silica ChromSep HPLC column SS (250 × 4.6 mm; Varian BV, Amsterdam, The Netherlands) with a ChromSep guard column. The mobile phase was hexane/1,4-dioxane (95:5, vol/vol), and the flow rate was 1.5 $mL.min^{-1}$. Samples (50 µL) were injected with a run time of 20 min at 25°C. Identification and quantification were made using standards of α-, β-, γ-, and δ-tocopherol (Sigma Ltd., Gillingham, United Kingdom). The CV of standards was acceptable at <4.1%.

Total phenolic content (TPC). Folin Ciocalteau reagent (0.25 mL) was allowed to react with methanol extract (50 µL) for 1 min. Sodium carbonate solution (20% wt/vol; 0.75 mL) was added, the sample vortexed and left for 1 min, then diluted to 5.0 mL with distilled water, vortexed, and left for 2 h. The sample was passed through a syringe filter (0.45 µm nylon filter) and the absorbance measured using a quartz cuvette at 760 nm with gallic acid as a standard.

RESULTS AND DISCUSSION

Oil bodies were recovered from sunflower seeds and washed with 10 mM sodium phosphate buffer, pH 7.5 (water-washed oil bodies: WWOB). Components of interest were measured in the parent seed and the WWOB (Table 1). Test samples were freeze-dried prior to tocopherol analysis to optimize the extraction of tocopherol with methanol (100%). It is clear from Table 1 that WWOB contain a significant level of tocopherol (38% of the seed tocopherol). Tocopherol in the residue represented 27% of the seed tocopherol, so the total recovery of tocopherol was 65%. This supports our hypothesis that tocopherol is integral with oil bodies. To test this hypothesis further, we carried out urea washing to remove extraneous proteins.

Table 1 shows that 17% of the seed weight can be extracted in one cycle of this aqueous oil body extraction; this equates to

TABLE 1
Extraction Yields of Lipid, Protein, Tocopherol, and Phenolics in Oil Bodies[a] Relative to Parent Seed

	Mass		Protein		Lipid		TPC[b]		Total tocopherol[c]		
	Mass (g)	Yield (%)	Mass (g)	Yield (%)	Mass (g)	Yield (%)	Mass (mg)	Yield (%)	Mass (mg)	Yield (%)	Mass (mg/g lipid)
Seed	47.5 ± 0.060	100	11.8 ± 0.94	100	12.9 ± 2.2	100	130 ± 3.9	100	4.66 ± 0.42	100	0.36
WWOB[d]	8.18 ± 1.5	17	2.04 ± 0.00	17	3.07 ± 0.32	24	9.00 ± 0.56	6.9	1.75 ± 0.20	38	0.57

[a]The values shown are means ± SD of three replicates, calculated on a dry weight basis.
[b]Total phenolic content (TPC) was determined by Folin Ciocalteau method (mg gallic acid equivalent).
[c]Total recovery of tocopherol is 65%. This is calculated from 100 × ([oil body] + [residue])/[seed], where [oil body], [residue], and [seed] are the total mass of tocopherol in the oil body, residue, and seed, respectively, and residue is the seed meal remaining after oil body extraction.
[d]Water-washed oil bodies (WWOB) for this study were recovered from one cycle of the extraction protocol from 47.5g seed.

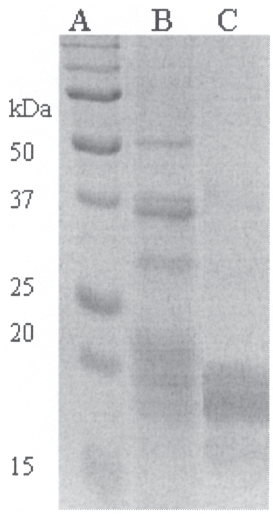

FIG. 1. SDS-PAGE of proteins from sunflower oil bodies. (A) Protein standards of molecular size as indicated; (B) water-washed oil body preparation; (C) urea-washed oil body preparation. All runs were performed on the same plate. Lane B indicates the large number of proteins associated with water-washed oil bodies; these residual proteins can be removed by urea washing. The effect of urea washing can be clearly observed in lane C, in which a urea-washed preparation is shown; the major remaining proteins are oleosin isoforms, which range from 18 to 22 kDa.

a lipid extraction yield of 24%. Repeat extractions showed a further release of oil bodies (data not shown), but a high extraction yield of oil bodies was not essential for this comparative study.

Interestingly, the total tocopherol extraction yield (38%) is significantly greater than the extraction yield of lipid (24%) and protein (17%). This suggests that tocopherol is either intrinsically associated with the oil bodies *in vivo*, and thus concentrated during oil body extraction, or passively associated with the proteins that bind weakly to the oil body as an artifact of their extraction. This increase in concentration of tocopherol during oil body extraction is further supported if we compare the total tocopherol concentration on a lipid basis, which increased from 0.36 mg/g in the seed to 0.57 mg/g in the oil body preparations. Nolasco *et al.* (13) reported a tocopherol concentration of 0.39 to 1.9 mg tocopherol·g oil^{-1} for intact sunflower seeds.

Tocopherol has a phenolic head group that has the potential to form hydrogen bonds with proteins that are extraneous or intrinsic to oil bodies. This is also true of the less hydrophobic phenolic compounds present in sunflower seeds; oil bodies were therefore washed with 9 M urea to remove extraneous proteins and to disrupt any hydrogen bonds between tocopherol or other phenolic compounds and intrinsic proteins. If our hypothesis is correct and tocopherol, unlike the less hydrophobic phenolic compounds, is an integral part of sunflower seed oil bodies, then one would predict a significant reduction in the TPC and relative increase in tocopherol of oil bodies if the 9 M urea-washing to remove extraneous proteins is effective. SDS-PAGE was used to monitor the removal of extraneous proteins (Fig. 1).

Urea removed most of the proteins from the isolated oil bodies apart from oleosin isoforms, which are intrinsically bound to their structure during oil body formation. This loss in residual proteins was associated with a significant reduction in TPC (Table 2). Tocopherol isoforms increased in concentration with purification of oil bodies, in contrast to TPC values. This supports the hypothesis that oilseed tocopherol is intrinsically bound to oil body structures and that oil body isolation offers a novel route for tocopherol extraction.

What remains unclear at this stage is how the tocopherol, known to be synthesized in plastids, becomes an intrinsic part of sunflower oil bodies. Perhaps, as suggested by Draper (14) and Horvath *et al.* (15), an extra-plastidic location of vitamin E synthesis exists, or an intracellular transport mechanism directs significant amounts of tocopherol to sunflower oil bodies.

The presence of tocopherol in sunflower seed oil bodies strongly suggests that it protects the PUFA in oil bodies against oxidation. This would support the suggestion made by Sattler and co-workers (5) that tocopherol would have to be associated with oil bodies to deliver the required level of antioxidant protection for the seed.

TABLE 2
Tocopherol and Total Phenolic Content[a] (TPC) of Sunflower Seed and Extracted Water-Washed (WWOB) and Urea-Washed Oil Bodies (UWOB)

	mg Tocopherol/kg		
	Seed	WWOB	UWOB
α-Tocopherol	92.7 ± 8.2	200 ± 23	368 ± 50
β-Tocopherol	4.64 ± 0.51	12.2 ± 1.6	21.7 ± 1.1
γ-Tocopherol	0.53 ± 0.053	1.24 ± 0.19	1.93 ± 0.23
δ-Tocopherol	0.30 ± 0.015	0.93 ± 0.15	ND
Total tocopherol	98.2	214	392
Isoform ratio α:β:γ:δ	94.4:4.7:0.5:0.3	93.3:5.7:0.6:0.4	94:5.5:0.5:0
	mg Gallic acid equiv/kg (dwb)		
	Seed	WWOB	UWOB
TPC	2743 ± 83	1066 ± 69	518.8 ± 110

[a]Values shown are means ± SD of three replicates calculated on a dry weight basis (dwb). ND, not detected.

ACKNOWLEDGMENTS

The authors wish to express their gratitude for financial support of this work by Firmenich UK and the Biotechnology and Biological Sciences Research Council.

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[Received May 18, 2005; accepted January 19, 2006]