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Antioxidant Levels in Germinating Soybean Seed Axes in Relation to Free Radical and Dehydration Tolerance[1]

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ABSTRACT

The axis of soybean seeds suffer dehydration injury if they are dried to 10% moisture at 36 hours of imbibition, but tolerate this stress if dried at 6 hours of imbibition. Deesterification of membrane phospholipids has been correlated with the increased permeability and increased lipid phase transition temperatures of membranes from dehydration injured tissues. Deesterification, measured as increased free fatty acid:phospholipid and decreased phospholipid:sterol ratios, occurred primarily when the tissue was in the dry state and did not change significantly ($P \leq 0.05$) with increasing imbibition time.

When liposomes were exposed to free radicals *in vitro*, wide angle x-ray diffraction indicated that the phase transition temperature of liposomes prepared from membrane lipid from 36-hour axes (susceptible) increased from 6 to 31°C. In contrast, those from membrane lipid from 6-hour axes (tolerant) increased from 3 to only 8°C, indicating that the tolerance of free radicals previously observed in these membranes was due to a lipid-soluble component.

Lipid-soluble antioxidants were detected in 6-hour imbibed axes in much greater quantities than in the 36-hour imbibed axes. The presence of lipid-soluble antioxidants in the membrane apparently contributes to the free radical tolerance of seed membranes observed during the early stages of germination, and these antioxidants may contribute to the dehydration tolerance of this tissue.

Seeds are generally tolerant of dehydration at maturity and during the early stages of germination, but once germination proceeds beyond a critical stage, they become susceptible to dehydration injury (17). As an example, soybean seeds tolerated dehydration, if they were dried to their original moisture content (10%) at any time up to 6 h after imbibition, but after 36 h of imbibition a similar dehydration treatment induced a total loss of viability (17). Germinating soybean seeds at these two extreme states, dehydration tolerant (6 h of imbibition) and dehydration susceptible (36 h of imbibition), have been employed to investigate the mechanisms of injury caused by dehydration stress. Increased rates of solute leakage, a symptom of membrane injury, were observed only in the axes but not in cotyledons (17, 18). Wide angle x-ray diffraction studies indicated that microsomal membranes isolated from dehydration-injured soybean axes contained gel phase lipid at physiological temperatures in addition to liquid crystalline lipid, a phenomenon which could explain the increased rate of cytoplasmic leakage (19). This increased liquid crystalline to gel phase transition temperature as observed in the dehydration-injured membranes was associated with an increased FFA[2] content and a decreased PL:S ratio suggesting that dehydration induced a deesterification of the membrane phospholipids (19). Deesterification of phospholipid is generally thought to be catalyzed by phospholipase but it may also proceed by a free radical-mediated mechanism (13). Attempts to simulate the dehydration injury to membranes *in vitro* by free radicals have indicated that free radicals are capable of inducing symptoms similar to that observed in dehydration injured membranes (20). Furthermore, membranes from dehydration-tolerant axes (at 6 h of imbibition) were less susceptible to free radical injury than those at 36 h (20). These observations lead to the hypothesis that the seed's tolerance of dehydration may be a result of the cellular membranes tolerance of free radicals (20). In this study we are attempting to identify mechanisms which could contribute to the free radical tolerance of the cellular membranes and its possible involvement in the dehydration tolerance in seeds.

MATERIALS AND METHODS

Membrane Isolation and Lipid Extraction. Soybean seeds (*Glycine max.* L. Merr. cv Maple Arrow) were imbibed for 6 or 36 h and, where applicable, were dehydrated/rehydrated as previously described (17). Smooth microsomal membranes were routinely isolated from fully hydrated soybean axes as described previously (7, 8, 19). Total lipids were extracted from the smooth microsomal fraction according to the procedure of Nichols (12). Phospholipid content of the lipid extract was determined as inorganic phosphate after $HClO_4$ digestion (3). Free fatty acids and S were separated by TLC and quantified by GC (19).

Time Course of Phospholipid Degradation. Axes from soybean seeds were imbibed for 36 h; dehydrated to 10% moisture; and rehydrated in distilled H_2O for 0, 0.5, 2, and 4 h. Smooth microsomal membranes were isolated from the dehydrated/rehydrated axes and also from 36-h imbibed nondehydrated axes. Phospholipid, FFA, and S contents of the membrane lipid fractions were quantified.

Liposome Preparation and Exposure to Free Radicals. Liposomes were prepared from total lipid extracts according to the basic procedure described previously (2). The lipid extract from the smooth microsomal fraction was evaporated to dryness under N_2. Residual solvent was removed in a vacuum desiccator and 10 ml of 100 mM NaCl was added to the dried lipid sample and shaken with glass beads. The suspension was centrifuged at 165,000*g* for 1.5 h to isolate liposomes.

The liposomes were resuspended in 5 ml of 10 μM xanthine-50 mM $NaHCO_3$ in a centrifuge tube. Continual mixing of the suspension was achieved using a stirring magnet, and 20 μl

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[2] Abbreviations: FFA, free fatty acid(s); PL, phospholipid(s); S, sterol(s).

containing 0.5 units of xanthine oxidase (Sigma) was added to the liposome suspension. Another 5 ml of substrate was gradually added to the reaction mixture during a 30-min period, then centrifuged at 165,000*g* for 1.5 h to obtain the liposome pellet. An identical sample was resuspended in 50 mM $NaHCO_3$ without xanthine but with the added enzyme as a control.

To examine the lipid phase properties of the free radical-treated liposomes, hydrated liposomes were transferred into a fine quartz capillary tube and left in a vacuum desiccator overnight to remove excess water. Subsequently, the capillary tube was sealed and placed in a temperature-controlled chamber and wide angle x-ray diffraction patterns were recorded as previously described (7, 8, 19).

Antioxidant Extractions and Assay. Samples of soybean axes were homogenized in 19 ml of chloroform-methanol-H_2O (5:10:4 by volume) using a polytron homogenizer. The suspension was again homogenized after addition of 1.5 ml chloroform and 1.5 ml of H_2O and was centrifuged at 10,000*g* for 10 min; the supernatant was taken and partitioned with 0.7% (w/v) NaCl to remove non-lipid material. The organic layer was evaporated to dryness *in vacuo* and residual lipid was resuspended in 3 ml ethanol and stored under N_2 at −20°C until required. Total lipid was quantified in an aliquot of the sample gravimetrically after evaporating the solvent, and all the samples were adjusted to the same lipid concentration (w/v) in ethanol.

The relative quantity of antioxidants in the tissue extract was determined by monitoring the inhibition of linoleic acid oxidation using a modification of the method of McKersie *et al.* (5). An emulsion was formed by mixing 0.2 ml of 0.2 M linoleic acid in ethanol, with 0.1 ml of either the sample extract in ethanol (800 μg lipid), ethanol alone (control), or α-tocopherol (standard) and then adding 3 ml of 10 mM KH_2PO_4 at pH 6.8. The reaction was initiated at 37°C by the addition of 1.5 ml 0.2 mM $FeSO_4$. An aliquot of the emulsion was taken immediately after addition of $FeSO_4$ and after a specific time of incubation, and 2 ml of 0.01 N NaOH in ethanol was added to clear the emulsion. Oxidation of linoleic acid was monitored by measuring the *A* at 232 nM. Per cent inhibition of the reaction was calculated as $[(OD_{232}\text{ control} - OD_{232}\text{ sample})/OD_{232}\text{ control}] \times 100$. A standard curve for per cent inhibition by α-tocopherol was constructed and relative quantity of antioxidants in the samples were calculated from the standard curve as α-tocopherol equivalents.

RESULTS AND DISCUSSION

The deesterification of membrane PL observed in dehydration of soybean axes (19) may be a consequence of enhanced phospholipase activity or nucleophilic attack by free radicals. The time-course study revealed that the majority of the PL degradation occurred when the axes were in the dry state (Table I). Although there seems to be a trend of increasing FFA levels with increasing reimbibition time up to 4 h, these differences were not statistically significant at $P \leq 0.05$. Similarly, the molar PL:S ratio of the smooth microsomal membranes from all dehydrated axes, regardless of the length of imbibition period, was approximately half that of the membranes from nondehydrated axes (Table I). There were no significant differences in the ratio during reimbibition. It appears from these data that PL deesterification predominately occurred prior to reimbibition. Furthermore, membrane injury in soybean axes occurred only when dehydrated below 20% moisture (17), and this threshold seems to be common to other seeds such as *Lotus corniculatus* L. and *Avena fatua* L. (9). It seems unlikely that an increase in hydrolytic enzyme activity would occur at such low water content. Membrane isolation was carried out at 0 to 4°C, a temperature range at which enzyme activity would be expected to be minimal. Granted the assumption that phospholipase activity would be minimal at low water contents and low temperatures, the time profile of PL deesterification is inconsistent with what might be expected of a strictly enzymic process.

Table I. *Free Fatty Acid:Phospholipid Ratio and Phospholipid:Sterol Ratio of Smooth Microsomal Membranes from Soybean Axes Dehydrated after 36 Hours of Imbibition and Reimbibed*

Values in a column followed by the same letter are not significantly different at $P \leq 0.05$ according to LSD test.

Dehydration Treatment	Reimbibition Period	FFA:PL Ratio	PL:S Ratio
	h	*mol:mol*	
Nondehydrated	—	0.03 b	38 a
Dehydrated	0	0.20 a	21 b
	0.5	0.20 a	20 b
	2.0	0.34 a	16 b
	4.0	0.31 a	19 b

Dehydration injury, observed as an increased lipid phase transition temperature, increased FFA:PL ratio, and decreased PL:S ratio in the membranes, can be simulated by exposing isolated membranes to free radicals *in vitro* (20). Membranes from axes in the dehydration tolerant state (6 h of imbibition) were less susceptible to this type of injury than those from axes imbibed for 36 h (20). Although not definitive, these data and the time course experiment imply that the membrane injury associated with dehydration in soybean axes is mediated by free radicals, as opposed to a strictly enzymic mechanism. Therefore, subsequent experiments were designed to reveal possible mechanisms of tolerance in these membranes to free radicals.

Wide angle x-ray diffraction patterns of liposomes which were prepared from the microsomal membrane lipid of 6-h imbibed and 36-h imbibed axes, but which were not exposed to free radicals, displayed a broad diffuse reflection with a Bragg spacing of 4.6 Å, when recorded at room temperature (Fig. 1, a and c). The diffraction pattern was similar when liposomes were prepared from membranes of 6-h imbibed axes and exposed to free

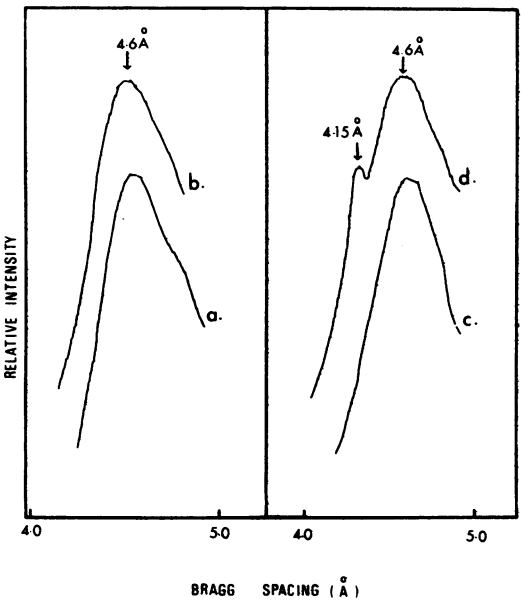

FIG. 1. Densitometer tracing of wide angle x-ray diffraction patterns, recorded at 25°C, of liposomes prepared from microsomal membranes. (a), Liposomes from membranes of 6-h imbibed axes; (b), liposomes from membranes of 6-h imbibed axes and exposed to free radicals; (c), liposomes from membranes of 36-h imbibed axes; (d), liposomes from membranes of 36-h imbibed axes and exposed to free radicals.

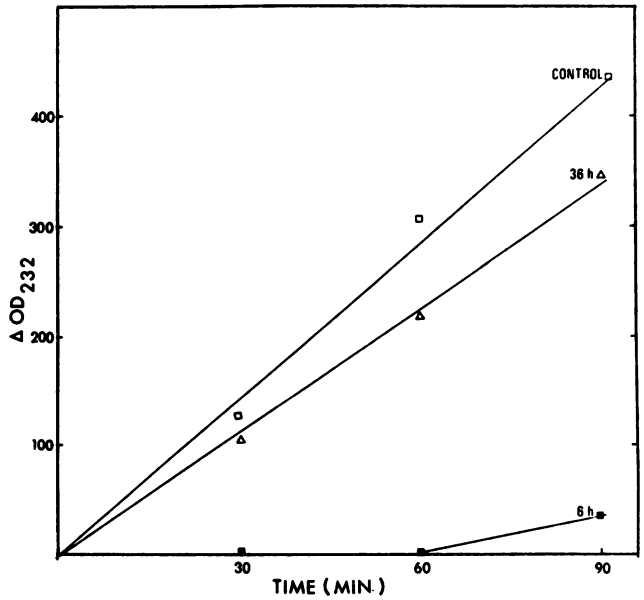

FIG. 2. Time course of Fe^{2+}-catalyzed oxidation of linoleic acid. Treatments are control, or with addition of 0.1 ml of lipid extract from 6-h imbibed or 36-h imbibed soybean axes. Lipid extracts contained the same concentration of lipid (w/v).

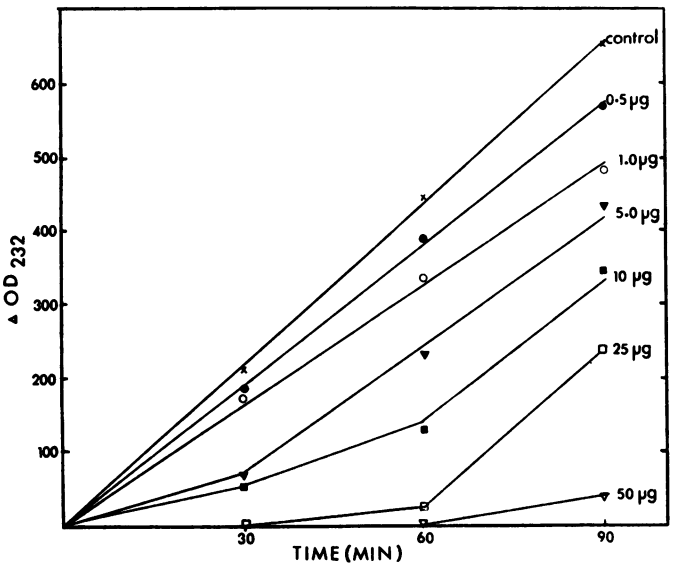

FIG. 3. Time course of Fe^{2+}-catalyzed linoleic acid oxidation with different concentration of α-tocopherol.

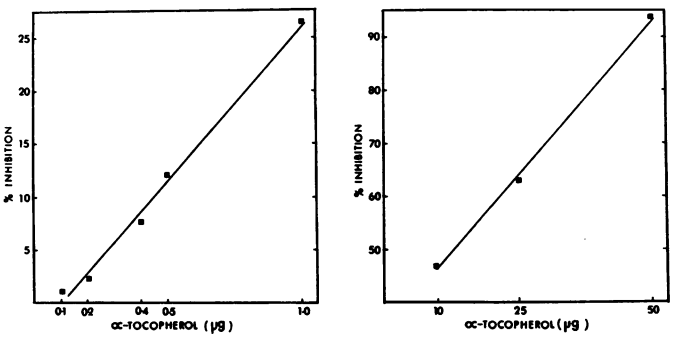

FIG. 4. Inhibition of linoleic acid oxidation at 90 min by different concentrations of α-tocopherol.

Table II. *Antioxidant Levels of Nondehydrated and Dehydrated Soybean Axes*

Antioxidant levels were calculated from the standard curve (Fig. 3) by the percentages shown. Values followed by the same letter are not significantly different at $P \leq 0.05$ according to LSD test.

Imbibition Period	Treatment	Inhibition of Linoleic Acid Oxidation	Antioxidants Tocopherol Equivalents
h		%	*mg g^{-1} lipid*
0	Not imbibed	81	49.9 b
6	Nondehydrated	87	56.6 a
	Dehydrated	83	52.0 b
36	Nondehydrated	15	0.7 c
	Dehydrated	2	0.1 c

radicals (Fig. 1b). The observed broad diffuse band derives from lipid in the liquid-crystalline state and is essentially disordered (7, 8, 14, 19). In contrast, when liposomes were prepared from membrane lipid of 36-h imbibed axes and subsequently exposed to free radicals, the x-ray diffraction pattern displayed a sharp band at 4.15 Å in addition to diffuse pattern at 4.6 Å (Fig. 1d). This sharp band represents lipid in the gel phase (7, 8, 14). Formation of gel phase lipid and increased lipid phase transition temperatures have been used to detect membrane deterioration in plant tissues after dehydration stress (19), senescence (1, 7, 8), paraquat treatment (1), exposure to ozone (14), and free radicals (20).

The lipid phase transition temperature of these liposome preparations was quantified as the highest temperature at which gel phase lipid was detected. The phase transition temperature of liposomes from membranes of 6-h axes increased from 3 to only 8°C as a result of exposure to free radicals, whereas a similar treatment of liposomes prepared from membranes of the 36-h axes increased the transition from 6 to 31°C. The response of liposomes to free radical exposure is essentially the same as the response of the respective membranes to free radicals (20). Therefore, the mechanism(s) of tolerance to free radicals in membranes from 6-h axes are, at least in part, associated with the lipid fraction. Such mechanisms may include the presence of lipid soluble antioxidants such as α-tocopherol, or phenolic antioxidants which are associated with membrane lipid (10, 11) and are capable of preventing lipid damage by scavenging free radicals (16). Formation of gel phase lipid in the microsomal membranes of *Phaseolus vulgaris* by exposure to ozone is mediated by free radicals and can be prevented by addition of antioxidants (15). Therefore, the subsequent experiment quantified the lipid-soluble antioxidant levels in these tissues.

Antioxidant content of the lipid fraction was estimated by the ability to inhibit Fe^{2+}-catalyzed linoleic acid oxidation. The oxidation of linoleic acid progressed at a linear rate for at least 90 min as indicated by OD_{232} (Fig. 2). The addition of a lipid extract from 6-h imbibed or 36-h imbibed axes or α-tocopherol (Fig. 3) inhibited the oxidation reaction. Increasing the concentration of α-tocopherol in the emulsion increased the inhibition (Fig. 3). Inclusion of α-tocopherol in higher quantities, *i.e.* 50 µg, totally inhibited the reaction up to 60 min and then the reaction rate increased (Fig. 3) presumably as the majority of the α-tocopherol was reduced. This time course pattern was observed after the addition of the lipid extract from 6 or 36-h imbibed axes (*cf.* Figs. 2 and 3). The relationship between per cent inhibition at 90 min and α-tocopherol concentration was used as standard (Fig. 4) to estimate the quantity of antioxidants present in the lipid extracts from the tissues, and was expressed

as α-tocopherol equivalents.

Mature dry axes and axes at 6 h of imbibition contained very high concentrations of antioxidants (Table II). Even after dehydration, at 6 h of imbibition the antioxidant content was as high as 52 mg g^{-1} lipid. In contrast, 36-h imbibed axes contained only trace amounts of antioxidants.

In these experiments, we have not investigated the free radical scavenging capacity of the membrane protein fraction. Superoxide dismutase is the most notable example of an enzyme which scavenges free radicals (4). Superoxide dismutase however, is associated with the cytoplasm and would not explain the observed differences in free radical tolerance of isolated membranes, and liposomes prepared from these membranes. The difference in quantities of lipid-soluble antioxidants between 6- and 36-h imbibed axes may account for the difference in the tolerance of respective membranes. Free radical-sensitive membranes from 36-h axes presumably do not contain sufficient quantities of antioxidants to scavenge free radicals and are, therefore, not protected from free radical injury. If the dehydration injury in soybean axes is mediated via free radicals as suggested (20), the dehydration tolerance of the seed in the early stages of germination may, at least in part, be related to the presence of large quantities of lipid-soluble antioxidants in the tissue.

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